



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

REC'D 13 OCT 2004

WIPO

PCT

Bescheinigung

Certificate

Attestation

Die angehefteten Unterla-
gen stimmen mit der
ursprünglich eingereichten
Fassung der auf dem näch-
sten Blatt bezeichneten
europäischen Patentanmel-
dung überein.

The attached documents
are exact copies of the
European patent application
described on the following
page, as originally filed.

Les documents fixés à
cette attestation sont
conformes à la version
initialement déposée de
la demande de brevet
européen spécifiée à la
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03077811.2

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Best Available Copy

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 03077811.2
Demande no:

Anmeldetag:
Date of filing: 05.09.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

CropDesign N.V.
Technologiepark 3
9052 Zwijnaarde
BELGIQUE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Plants having modified growth characteristics and method for making the same

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12Q/

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

103-CDKB-PROV

Plants having modified growth characteristics and method for making the same

The present invention relates generally to the field of molecular biology and concerns a method for modifying plant growth characteristics. More specifically, the present invention concerns a method for modifying plant growth characteristics by modulating expression in a plant of a B-type CDK (cyclin dependent kinase) nucleic acid and/or by modulating activity and/or levels in a plant of a B-type CDK protein. The present invention also concerns plants having modulated expression of a B-type CDK nucleic acid and/or modulated activity and/or levels of a B-type CDK protein, which plants have modified growth characteristics relative to corresponding wild type plants. The present invention also provides a novel screening method for the identification of mutant CDKs having enhanced CDK activity relative to corresponding non-mutated CDKs. The present invention also provides a novel screening method for the identification of non-active CDKs that are still able to bind to CKIs (cyclin dependent kinase inhibitors). The invention also provides mutant CDKs obtainable by the screening methods according to the invention.

The ever-increasing world population and the dwindling supply of arable land available for agriculture fuel agricultural research towards improving the efficiency of agriculture. Conventional means for crop and horticultural improvements utilise selective breeding techniques to identify plants having desirable characteristics. However, such selective breeding techniques have several drawbacks, namely that these techniques are typically labour intensive and result in plants that often contain heterogeneous genetic components that may not always result in the desirable trait being passed on from parent plants. Advances in molecular biology have allowed mankind to modify the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has the capacity to deliver crops or plants having various improved economic, agronomic or horticultural traits. A trait of particular economic interest is yield. Yield is normally defined as the measurable produce of economic value from a crop. This may be defined in terms of quantity and/or quality. Crop yield is influenced by the typical stresses to which plants or crops are subjected. Such stresses include environmental (abiotic) stresses (such as temperature stresses caused by atypical high or low temperatures; stresses caused by nutrient deficiency; stresses caused by lack of water (drought)) and biotic stresses (which can be imposed on plants by other plants (weeds), animal pests and pathogens). Crop yield may not only be increased by combating one or more of the stresses to which the crop or

103-CDKB-PROV

plant is subjected, but may also be increased by modifying the inherent growth mechanisms of a plant.

5 The inherent growth mechanisms of a plant reside in a highly ordered sequence of events collectively known as the 'cell cycle'. Progression through the cell cycle is fundamental to the growth and development of all multicellular organisms and is crucial to cell proliferation. The major components of the cell cycle are highly conserved in yeast, mammals, and plants. The cell cycle is typically divided into the following sequential phases: G0 – G1 – S – G2 – M. DNA replication or synthesis generally takes place during the S phase ("S" is for DNA synthesis) and mitotic segregation of the chromosomes occurs during the M phase (the "M" is for mitosis), 10 with intervening gap phases, G1 (during which cells grow before DNA replication) and G2 (a period after DNA replication during which the cell prepares for division). Cell division is completed after cytokinesis, the last step of the M phase. Cells that have exited the cell cycle and that have become quiescent are said to be in the G0 phase. Cells in this phase can be stimulated to reenter the cell cycle at the G1 phase. The "G" in G1, G2 and G0 stands for "gap". 15 Completion of the cell cycle process allows each daughter cell during cell division to receive a full copy of the parental genome.

20 Cell division is controlled by two principal cell cycle events, namely initiation of DNA synthesis and initiation of mitosis. Each transition to each of these key events is controlled by a checkpoint represented by specific protein complexes (Involved in DNA replication and division). The expression of genes necessary for DNA synthesis at the G1/S boundary is regulated by the E2F family of transcription factors in mammals and plant cells (La Thangue, 1994; Muller *et al.*, 2001; De Veylder *et al.*, 2002). Entry into the cell cycle is 25 regulated/triggered by an E2F/Rb complex that integrates signals and allows activation of transcription of cell cycle genes. The transition between the different phases of the cell cycle, and therefore progression through the cell cycle, is driven by the formation and activation of different heterodimeric serine/threonine protein kinases, generally referred to as cyclin-dependent kinases (CDKs). A prerequisite for activity of these kinases is the physical 30 association with a specific cyclin, the timing of activation being largely dependent upon cyclin expression. Cyclin binding induces conformational changes in the N-terminal lobe of the associating CDK and contributes to the localisation and substrate specificity of the complex. Monomeric CDKs are activated when they are associated with cyclins and thus have kinase activity. Cyclin protein levels fluctuate in the cell cycle and therefore represent a major factor 35 in determining timing of CDK activation. The periodic activation of these complexes containing cyclins and CDK during cell cycle mediates the temporal regulation of cell-cycle transitions (checkpoints). Other factors regulating CDK activity include CDK inhibitors (CKIs or ICKs,

103-CDKB-PROV

KIPs, CIPs, INKs), CDK activating kinases (CAKs), a CDK phosphatase (Cdc25) and a CDK subunit (CKS) (Mironov *et al.* 1999; Reed 1996).

In plants, two major classes of CDKs, known as A-type and B-type CDKs, have been studied to date. The A-type CDKs regulate both the G1-to-S and G2-to-M transitions, whereas the B-type CDKs seem to control the G2-to-M checkpoint only (Hemerly *et al.*, 1995; Magyar *et al.*, 1997; Porceddu *et al.*, 2001). In addition, the presence of C-type CDKs and CDK-activating kinases (CAKs) has been reported (Magyar *et al.*, 1997; Umeda *et al.*, 1998; Joubès *et al.*, 2001), as has the presence of D-type, E-type and F-type CDKs (Vandepoele *et al.* 2002).

The ability to influence the cell cycle in a plant (either using recombinant DNA technology or using non-recombinant means), and to thereby modify various growth characteristics of a plant, would have many applications in areas such as crop enhancement, plant breeding, production of ornamental plants, arboriculture, horticulture, forestry, the production of algae or plants (for example for use as bioreactors, for the production of substances such as pharmaceuticals, antibodies, or vaccines, or for the bioconversion of organic waste or for use as fuel in the case of high-yielding algae and plants).

It has now been found that modulating expression in a plant of a B-type CDK nucleic acid and/or modulating activity and/or levels in a plant of a B-type CDK protein gives plants having modified growth characteristics. Therefore according to a first embodiment of the present invention there is provided a method for modifying the growth characteristics of a plant, comprising modulating expression in a plant of a B-type CDK nucleic acid and/or modulating activity and/or levels in a plant of a B-type CDK protein.

Modulating (enhancing or decreasing) expression of a B-type CDK nucleic acid encompasses altered expression of a B-type CDK gene. Modulating (enhancing or decreasing) activity and/or levels of a B-type CDK protein encompasses altered activity and/or levels of a gene product, namely a B-type CDK polypeptide. The altered expression, activity and/or levels are altered compared to expression, activity and/or levels of a B-type CDK in corresponding wild-type plants. The modulated gene expression may result from altered expression levels of an endogenous B-type CDK gene and/or may result from altered expression levels of a B-type CDK nucleic acid/gene previously introduced into a plant. Similarly, modulated levels and/or activity of a B-type CDK protein may be due to modulated expression of an endogenous B-type CDK nucleic acid/gene and/or due to modulated expression of a B-type nucleic acid/gene previously introduced into a plant. Modulating expression of a gene/nucleic acid and/or

103-CDKB-PROV

modulating activity and/or levels of a gene product may be effected, for example, by chemical means and/or recombinant means.

- 5 Chemical means for the modulation of expression of a B-type CDK nucleic acid and/or for the modulation of activity and/or levels of a B-type CDK protein, include for example the exogenous application of one or more compounds or elements capable of modulating activity and/or levels of a B-type CDK protein and/or expression of a B-type CDK nucleic acid. The exogenous application may take place on wild-type plants or on transgenic plants that have previously been transformed with a B-type CDK nucleic acid/gene or that have previously been transformed with any other transgene. The term "exogenous application" taken in its broadest context includes the contacting or administering of a suitable compound or element to plant cells, tissues, organs or to the whole organism. The compound or element may be exogenously applied to a plant in a form suitable for plant uptake (such as through application to the soil for uptake via the roots, or in the case of some plants by applying directly to the leaves, for example by spraying).
- 10
- 15

- Suitable compounds or elements for exogenous application include B-type CDK proteins or B-type CDK nucleic acids. Similarly, homologues, derivatives or active fragments of B-type CDK proteins and/or fragments or sequences capable of hybridizing with B-type CDK nucleic acids may also be used. The exogenous application of compounds or elements capable of decreasing levels of factors that directly or indirectly activate a B-type CDK protein will also be suitable in practising the invention. Such factors include, for example, cyclin dependent kinase inhibitors (CKIs). Also included are antibodies that can recognise or mimic the function of B-type CDK proteins. Such antibodies may comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies, as well as fragments thereof. Additionally or alternatively, the resultant effect may also be achieved by the exogenous application of an interacting protein or by the exogenous application of an activator of the gene/gene product. Additionally or alternatively, the compound or element may be a mutagenic substance, such as a chemical selected from any one or more of: N-nitroso-N-ethylurea, ethylene imine, ethyl methanesulphonate and diethyl sulphate. Mutagenesis may also be achieved by exposure to ionising radiation, such as X-rays or gamma-rays or ultraviolet light. Methods for introducing mutations and for the testing of the effects of the mutations (such as by monitoring gene expression and/or protein activity) are well known in the art.
- 20
- 25
- 30

- 35 Therefore, according to one feature of the present invention, there is provided a method for modifying the growth characteristics of a plant, comprising exogenous application of one or

103-CDKB-PROV

more compounds or elements capable of modulating in a plant expression of a B-type CDK nucleic acid and/or modulating in a plant the activity and/or levels of a B-type CDK protein.

5 Additionally or alternatively, and according to a preferred feature of the present invention, modulation of expression of a B-type CDK nucleic acid and/or modulation of activity and/or levels of a B-type CDK protein may be effected by recombinant means. Such recombinant means may comprise a direct and/or indirect approach.

10 For example, an indirect recombinant approach may comprise introducing, into a plant, a nucleic acid capable of modulating activity and/or levels of the protein in question (a B-type CDK protein) and/or expression of the gene in question (a gene encoding a B-type CDK protein).

15 Examples of such nucleic acids to be introduced into a plant include nucleic acids encoding transcription factors or activators or inhibitors that can modulate expression of a B-type CDK nucleic acid or that modulate the activity and/or levels of a B-type CDK protein. For example, in order to increase levels of a CDK B1;1 in a plant, increasing levels of, for example, an E2F transcription factor may be envisaged.

20 Also encompassed by an indirect approach for modulating activity and/or levels of a B-type CDK protein and/or expression of a B-type CDK gene is the inhibition or stimulation of regulatory sequences (such as promoters) that drive expression of a native B-type CDK gene or that of a corresponding transgene previously introduced into a plant. Such regulatory sequences may be introduced into a plant.

25 Furthermore, modulation of the activity of a B-type CDK protein may be effected by altering levels in a plant of a factor capable of interacting with a B-type CDK protein. Such factors include ligands of the B-type CDK protein (regulators, subunits, substrates or targets), such as A-type, B-type or D-type cyclins, CDK activating kinases (CAK), CDK subunits (CKS) and CDK
30 inhibitors (CKI, KRP).

The nucleic acid to be introduced into a plant may be derived from any source provided that that nucleic acid, when expressed in a plant, leads to modulated expression of a B-type CDK gene or modulated activity and/or levels of a B-type CDK protein. The nucleic acid to be
35 introduced into a plant may be isolated from a microbial source, such as bacteria, yeast or fungi, or from a plant, algae or animal (including human). This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through

103-CDKB-PROV

deliberate human manipulation. The nucleic acid sequence is preferably a homologous nucleic acid sequence, i.e. a nucleic acid sequence obtained from a plant, whether from the same plant species or different. Modulated B-type CDK expression and/or activity and/or levels in a plant may be due to a nucleic acid previously introduced into the plant and/or may be due to an endogenous gene.

Therefore, according to another feature of the present invention, there is provided a method for modifying the growth characteristics of a plant, comprising introducing, into a plant, a nucleic acid capable of modulating activity and/or levels of a B-type CDK protein and/or capable of modulating expression of a B-type CDK gene, which nucleic acid is selected from:

- (i) Nucleic acids encoding transcription factors, activators or inhibitors of a B-type CDK;
- (ii) B-type CDK regulatory sequences; and
- (iii) Ligands of a B-type CDK protein.

15

A direct and preferred approach on the other hand, for modifying the growth characteristics of plants, comprises introducing into a plant a B-type CDK nucleic acid, which nucleic acid preferably encodes a B-type CDK protein. The nucleic acid may be introduced into a plant by, for example, transformation. Therefore, according to a preferred aspect of the present invention, there is provided a method for modifying the growth characteristics of a plant comprising introducing into a plant, in an expressible format, a B-type CDK nucleic acid, which B-type nucleic acid preferably encodes a B-type CDK protein.

The term "B-type CDK nucleic acid" as defined herein encompasses any nucleic acid sequence which when used in the construction of a CDK phylogenetic tree, such as the one depicted in Fig. 1, tends to cluster around the B-type CDKs rather than any of the other CDKs. A person skilled in the art could readily determine whether any nucleic acid sequence in question falls within the definition of a "B-type CDK nucleic acid" using known techniques and software for the making of such phylogenetic trees, such as a GCG, EBI or CLUSTAL package, using default parameters. Upon construction of such a phylogenetic tree, sequences clustering in the B-type CDK group will be considered to fall within the definition of a "B-type CDK" as used herein and will therefore be useful in performing the methods of the invention.

The term "B-type CDK amino acid" as defined herein encompasses any amino acid sequence which when used in the construction of a CDK phylogenetic tree, such as the one depicted in Fig. 1, tends to cluster around the B-type CDKs rather than any of the other CDK groups. A person skilled in the art could readily determine whether any amino acid sequence in question

103-CDKB-PROV

falls within the definition of a "B-type CDK amino acid" using known techniques and software for the making of such a phylogenetic tree, such as a GCG, EBI or CLUSTAL package, using default parameters. Upon construction of such a phylogenetic tree, sequences clustering in the B-type CDK group will be considered to fall within the definition of a "B-type CDK" and will therefore be useful in performing the methods of the invention. A "B-type CDK amino acid" as defined herein may also be identified by the presence of a PPTALRE motif.

The B-type CDK nucleic acid may be derived (either directly or indirectly (if subsequently modified)) from any source provided that that nucleic acid, when expressed in a plant, leads to modulated B-type CDK gene expression and/or modulated levels and/or activity of a B-type CDK protein. The B-type CDK nucleic acid/gene may be isolated or derived from a microbe, such as bacteria, yeast or fungi, or from a plant, an algae or an animal (including human). This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. The nucleic acid sequence is preferably a homologous nucleic acid sequence, i.e. a nucleic acid sequence obtained from a plant, whether from the same plant species or different. The B-type CDK nucleic acid may be isolated from a monocotyledonous or dicotyledonous species, preferably from the family *Brassicaceae*, further preferably from *Arabidopsis thaliana*. The nucleic acid is preferably a class 1 B-type CDK, such as a class 1 B-type CDK selected from the examples of class 1 CDKs shown in Fig. 1, namely, CDK B1;1 from *Arabidopsis thaliana*, CDK B1;2 from *Arabidopsis thaliana*, CDKB1;1 from *Lycopersicon esculentum* (tomato), CDK B1;1 from *Antirrhinum majus*, CDK B1;1 from *Medicago sativa* (alfalfa) and CDK B1 from *Dunaliella tertiolecta*, further preferably the class 1 B-type CDK is a CDK B1;1 from *Arabidopsis thaliana* or a CDK B1;2 from *Arabidopsis thaliana*. Alternatively, the nucleic acid is preferably a class 2 B-type CDK, such as a class 2 B-type CDK selected from the examples shown in Fig 1, namely, a CDK B2;1 from *Arabidopsis thaliana*, a CDK B2;2 from *Arabidopsis thaliana*, a CDK B2;1 from *Antirrhinum majus*, a CDK B2;1 from *Mesembryanthemum crassifolium*, a CDK B2;1 from *Medicago sativa*, a CDK B2;1 from *Lycopersicon esculentum* and a CDK B 1 from *Oryza sativa*, further preferably the class 2 B-type CDK is a CDK B2;2 from *Arabidopsis thaliana*.

30

Most preferably the CDK B1;1 nucleic acid is as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;1 protein is as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment thereof. Most preferably the CDK B1;2 nucleic acid is as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;2 protein is as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof. Most preferably the CDK B2;2 nucleic acid is as represented by SEQ ID NO:

35

103-CDKB-PROV

5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B2;2 protein is as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof. Each of the CDK B1;1, CDKB1;2 and CDK B2;2 nucleic acids/proteins also encompass the variant nucleic acids and amino acids as described hereinafter.

Although the invention has been exemplified with a B-type CDK according to SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5, and corresponding amino acids according to SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6, respectively, it would be apparent to a person skilled in the art that the methods according to the invention may also be practised using variant nucleic acids and variant amino acids, such as the ones defined hereinafter. Therefore, taken in a broad context, the term "B-type CDK" protein/nucleic acid also encompasses variant nucleic acids and variant amino acids suitable for practising the methods according to the invention. Preferably, variant nucleic acids and variant amino acids suitable for practising the methods according to the invention include those falling within the definition of a "B-type CDK", meaning that upon construction of a phylogenetic tree, such as the one depicted in Fig. 1, the variant sequences of interest would tend to cluster around the B-type CDKs.

Suitable variant nucleic acid and amino acid sequences useful in practising the method according to the invention, include:

- (i) Functional portions of a B-type CDK nucleic acid/gene;
- (ii) Sequences capable of hybridising with a B-type CDK nucleic acid/gene;
- (iii) Alternative splice variants of a B-type CDK nucleic acid/gene;
- (iv) Allelic variants of a B-type CDK nucleic acid/gene;
- (v) Homologues, derivatives and active fragments of a B-type CDK protein;
- (vi) Mutant B-type CDKs;

An example of a variant B-type nucleic acid/gene is a functional portion of a B-type nucleic acid/gene. It would be apparent to a person skilled in the art that the full length DNA sequence is not a prerequisite to carrying out the methods according to the invention. The methods according to the invention may advantageously be practised using functional portions of a B-type CDK. A functional portion refers to a piece of DNA derived or prepared from an original (larger) DNA molecule, which DNA portion, when introduced and expressed in a plant, gives plants having modified growth characteristics. The portion may comprise many genes, with or without additional control elements or may contain spacer sequences. The portion may be made by making one or more deletions and/or truncations to the nucleic acid sequence of, for example, any one of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5. Techniques for

103-CDKB-PROV

Introducing truncations and deletions into a nucleic acid are well known in the art. Portions suitable for use in the methods according to the invention may readily be determined using routine techniques, such as by assaying for B-type CDK activity and/or by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the portion to be tested for functionality.

An example of a further variant B-type CDK nucleic acid is a sequence that is capable of hybridising to a B-type CDK. Advantageously, the methods according to the present invention may also be practised using sequences capable of hybridising to a B-type CDK, particularly a B-type CDK as represented by any one of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5, which hybridising sequences are preferably those falling within the definition of a "B-type CDK", meaning that upon construction of a phylogenetic tree, such as the one depicted in Fig. 1, the hybridising sequence would be one that tends to cluster around the B-type CDKs rather than any of the other CDK groups. Hybridising sequences suitable for use in the methods according to the invention may readily be determined using routine techniques, such as by assaying for B-type CDK activity and/or by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the hybridising sequence.

The term "hybridisation" as defined herein is a process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to e.g. a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, *in situ* hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is

103-CDKB-PROV

- influenced by conditions such as temperature, salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na₃-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York, but the skilled craftsman will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Sufficiently low stringency hybridisation conditions are particularly preferred (at least in the first instance) to isolate nucleic acids heterologous to the DNA sequences of the invention defined supra. An example of low stringency conditions is 4-6x SSC / 0.1-0.5% w/v SDS at 37-45°C for 2-3 hours. Depending on the source and concentration of the nucleic acid involved in the hybridisation, alternative conditions of stringency may be employed, such as medium stringency conditions. Examples of medium stringency conditions include 1-4x SSC / 0.25% w/v SDS at ≥ 45°C for 2-3 hours. An example of high stringency conditions includes 0.1-1x SSC / 0.1% w/v SDS at 60°C for 1-3 hours. The skilled man will be aware of various parameters which may be altered during hybridisation and washing and which will either maintain or change the stringency conditions. The stringency conditions may start low and be progressively increased until there is provided a hybridising B-type CDK nucleic acid, as defined hereinabove. Elements contributing to heterology include allelism, degeneration of the genetic code and differences in preferred codon usage.
- Another example of a variant B-type CDK is an alternative splice variant of a B-type CDK. The methods according to the present invention may also be practised using an alternative splice variant of a B-type CDK nucleic acid/gene. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid in which selected introns and/or exons have been excised, replaced or added. Such splice variants may be found in nature or can be manmade using techniques well known in the art. The splice variants useful in the methods according to the invention are preferably "B-type CDKs", meaning that upon construction of a phylogenetic tree, such as the one depicted in Fig. 1, the splice variant of interest would be one tending to cluster around the B-type CDKs rather than around any of the other CDK groups. Preferably, the splice variant is a splice variant of the sequence represented by any of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5. Splice variants suitable for use in the methods according to the invention may readily be determined using routine techniques, such as by assaying for B-

103-CDKB-PROV

type CDK activity and/or by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the splice variant.

Another example of a variant B-type CDK is an allelic variant. Advantageously, the methods according to the present invention may also be practised using allelic variants of a B-type CDK nucleic acid, preferably an allelic variant of a sequence represented by any of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these isolated natural alleles in the methods according to the invention. The allelic variants useful in the methods according to the invention are preferably "B-type CDKs", meaning that upon construction of a phylogenetic tree, such as the one depicted in Fig. 1, the allelic variant of interest would tend to cluster around the B-type CDKs. Allelic variants suitable for use in the methods according to the invention may readily be determined using routine techniques, such as by assaying for B-type CDK activity and/or by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the allelic variant.

The methods according to the present invention may also be practised by introducing into a plant at least a part of a (natural or artificial) chromosome (such as a Bacterial Artificial Chromosome (BAC)), which chromosome contains at least a B-type CDK gene/nucleic acid, optionally together with one or more related gene family members. Therefore, according to a further aspect of the present invention, there is provided a method for modifying the growth characteristics of plants by introducing into a plant at least a part of a chromosome comprising at least a B-type CDK gene/nucleic, which B-type CDK gene/nucleic is preferably one represented by any one of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5.

According to a preferred aspect of the present invention, enhanced or increased expression of a B-type CDK nucleic acid is envisaged. Methods for obtaining enhanced or increased expression of genes or gene products are well documented in the art and include, for example, overexpression driven by a strong promoter, the use of transcription enhancers or translation enhancers.

Examples of variant B-type amino acids include homologues, derivatives and active fragments of a B-type CDK protein. Advantageously, the methods according to the present invention may also be practised using homologues, derivatives or active fragments of a B-type CDK, preferably using homologues, derivatives or active fragments of a B-type CDK as represented by any one of SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6.

103-CDKB-PROV

"Homologues" of a B-type CDK protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α -helical structures or β -sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) *Proteins*. W.H. Freeman and Company). The homologues useful in the methods according to the invention are preferably B-type CDKs, meaning that upon construction of a phylogenetic tree, such as the one depicted in Fig. 1, any homologous sequences of interest would tend to cluster around B-type CDKs rather than any other group of CDKs. Preferably, such B-type CDKs have in increasing order of preference at least 60% or 65% or 70% or 75% or 80% or 85% or 90% or 95%, 96%, 97%, 98%, 99% or more sequence identity or similarity (functional identity) to an unmodified B-type CDK protein (which unmodified B-type CDK protein is preferably any one of the protein sequences represented SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6). Preferably, the homologue of a B-type CDK comprises at least a PPTALRE motif.

Two special forms of homology: orthologs and paralogs, are evolutionary concepts used to describe the ancestral relationships of genes. The term "paralogous" relates to gene duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The term "homologues" as used herein also encompasses paralogues and orthologues of the proteins useful in the methods according to the invention.

"Substitutional variants" of a protein are those in which at least one residue in an amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1 to 10 amino acid residues and deletions will range from about 1 to 20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

"Insertional variants" of a protein are those in which one or more amino acid residues are introduced into a predetermined site in a protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxy-terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or

103-CDKB-PROV

carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, (histidine)6-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag-100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

"Deletion variants" of a protein are characterised by the removal of one or more amino acids from the protein. Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. Methods for the manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen *in vitro* mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

Methods for the search and identification of B-type CDK homologues would be well within the realm of a person skilled in the art. Methods for the alignment of sequences for comparison are well known in the art. Such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 443-453, 1970) to find the alignment of two complete sequences that maximises the number of matches and minimises the number of gaps. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information.

The term "derivatives" refers to peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the protein, for example, as represented by any one of SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6. "Derivatives" of a B-type CDK protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which

103-CDKB-PROV

is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid sequence of a naturally-occurring protein.

5 "Active fragments" of a B-type CDK protein encompasses at least seven contiguous amino acid residues of a protein, which residues retain similar biological and/or functional activity to the naturally occurring protein. The active fragment preferably comprises at least a PPTALRE motif.

10 Further advantageously, the methods according to the present invention may also be practised using mutant CDKs, which mutant CDKs have at least a substantially similar, preferably enhanced, biological activity compared with corresponding wild-type CDK proteins.

15 The present invention further provides a hitherto unknown screening method for the identification of CDKs having enhanced CDK activity relative to corresponding non-mutated or wild-type CDKs. These CDK mutants having enhanced CDK activity may be used in the methods according to the invention or may find uses in other areas.

20 Therefore according to a second embodiment of the present invention, there is provided a screening method for the identification of mutant CDKs having substantially similar or enhanced CDK activity relative to corresponding non-mutated CDKs, which method comprises the steps of:

- (i) Providing CDK mutants;
- (ii) Identifying ICK non-reacting mutants;
- (iii) Identifying mutants having cyclin-binding activity; and optionally followed by,
- 25 (iv) A yeast complementation assay on resultant mutants from steps (ii) and (iii).

The novel screening method may be preceded with steps for mutating wild-type CDKs, if necessary. These steps comprise:

- (a) Providing wild-type CDK amino acids; and
- 30 (b) Mutating substantially each CDK amino acid at least at one amino acid position.

A mutation may be introduced into the CDK using conventional techniques, such as error prone PCR. The mutation may be introduced randomly or by site directed mutagenesis.

35 Although the method for the identification of CDK mutants has been exemplified with mutant CDK A-type proteins, the method is equally well suited to the identification of other mutant CDKs.

103-CDKB-PROV

Examples of particularly preferred mutants (mutants 1 to 3) obtainable by the above screening method according to the invention are listed in Table A below. The mutants are mutants of an A-type CDK, namely an A;1 CDK from rice (see SEQ ID NO: 8). These mutants may be of particular use in the methods for modifying the growth characteristics of plants using the novel methods as described hereinabove.

Alternatively, a CDK having a mutation consisting of at least one of the seven amino acid changes identified in Table A may also be useful in the methods according to the invention. The suitability of such a mutant in the methods according to the invention may readily be determined by passing the mutants through the novel screening method identified above so as to determine whether the mutant has substantially similar or enhanced CDK activity relative to corresponding non-mutated CDKs.

Table A: Mutants that bind to cyclin, but not to ICKs

Mutant	SEQ ID NO	Mutation Position		
1	SEQ ID NO: 9	Y4H	V79D	A152T
2	SEQ ID NO: 10	I30T		
3	SEQ ID NO: 11	E5V	R122S	K143E

The mutants are denoted by a change in the appropriate amino acid residue. In the case of mutant 1, for example, at position 4, the Y is substituted for an H; at position 79, the V is substituted for a D; and at position 152 the A is substituted for a T. Mutations positions are calculated from the first methionine of SEQ ID NO: 7.

Therefore according to another embodiment of the present invention, there is provided a method for modifying the growth characteristics of plants, comprising modulating, preferably increasing, activity and/or levels of a CDK mutant comprising at least one of the seven amino acid changes identified in Table A above. The mutated amino acid itself may be introduced directly into a plant cell or into the plant itself (including introduction into a tissue, organ or any other part of a plant).

Also of interest are CDKs with no activity but that are still able to bind to CKI, therefore constituting CKI traps. This hitherto unknown method comprises the steps of:

- (i) Providing CDK mutants;
- (ii) Identifying ICK binding mutants; and
- (iii) Identifying non-cyclin-binding mutants.

103-CDKB-PROV

The novel screening method may be preceded with steps for mutating wild-type CDKs, if necessary. These steps comprise:

- (a) Providing wild-type CDK amino acids; and
- (b) Mutating substantially each CDK amino acid at least at one amino acid position.

5

A mutation may be introduced into the CDK using conventional techniques, such as error prone PCR. The mutation may be introduced randomly or by site directed mutagenesis.

- 10 Particularly suitable mutant CDKs obtainable by the abovementioned screening method are listed in Table B below. The mutants are mutants of an A-type CDK, namely an A;1 CDK from *Arabidopsis thaliana* (see SEQ ID NO: 7).

Table B: Mutants that bind CKI but not cyclin

Mutant	SEQ ID NO	Mutant Position						
		G154R						
4	SEQ ID NO: 12	G154R						
5	SEQ ID NO: 13	Q3P	E38A	R137L	S182A	I193T	M267V	R279Q

15

Mutations positions are calculated from the first methionine of SEQ ID NO: 8.

According to a third embodiment of the present invention, there is provided an isolated CDK nucleic acid molecule comprising:

20

- (a) a nucleic acid encoding a CDK mutant represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13;

25

- (b) a nucleic acid encoding a homologue, derivative or active fragment of a CDK mutant represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, which homologue, derivative or active fragment preferably comprises at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;

30

- (c) a nucleic acid capable of hybridising with a nucleic acid of (a) or (b) above, wherein the hybridising sequence preferably encodes a protein comprising at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;
- (d) a nucleic acid of (a) to (c) above which is degenerate as a result of the genetic code;

103-CDKB-PROV

- (e) allelic variants of the nucleic acids of (a) to (d), which allelic variant preferably encodes a protein comprising at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B; and
- 5 (f) alternative splice variants of the nucleic acids of (a) to (e), which alternative splice variants preferably encode a protein comprising at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B.

10 According to a fourth embodiment of the present invention, there is provided a CDK mutant, comprising:

- (a) an amino acid represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13; and
- 15 (b) a fragment of an amino acid of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, which fragment preferably comprises at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B.

20 According to a fifth embodiment of the present invention, genetic constructs and vectors to facilitate introduction and/or expression of the nucleotide sequences useful in the methods according to the invention are provided. Therefore, according to a fifth embodiment of the present invention, there is provided a gene construct comprising:

- (i) a nucleic acid capable of modulating expression of a B-type CDK gene/nucleic acid and/or activity and/or levels of a B-type CDK protein; or
- 25 (ii) a nucleic acid encoding a CDK mutant, which CDK mutant comprises at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;
- (iii) one or more control sequences capable of driving expression of the nucleic acid of (i) or (ii); and optionally
- 30 (iv) a transcription termination sequence.

Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming

35 into plants and suitable for expression of the gene of interest in the transformed cells.

103-CDKB-PROV

The nucleic acid encoding a CDK mutant may be any of the mutant-encoding nucleic acids described hereinbefore.

- 5 The nucleic acid capable of modulating expression of a nucleic acid encoding a B-type CDK protein and/or activity of the B-type CDK protein itself may be any of the nucleic acids hereinbefore described. Preferably, the nucleic acid is a B-type CDK nucleic acid/gene. The B-type CDK nucleic acid may be isolated from a monocotyledonous or dicotyledonous species, preferably from the family *Brassicaceae*, further preferably from *Arabidopsis thaliana*. The nucleic acid is preferably a class 1 B-type CDK, such as a class 1 B-type CDK selected from
- 10 the examples of class 1 CDKs shown in Fig. 1, namely, CDK B1;1 from *Arabidopsis thaliana*, CDK B1;2 from *Arabidopsis thaliana*, CDKB1;1 from *Lycopersicon esculentum* (tomato), CDK B1;1 from *Antirrhinum majus*, CDK B1;1 from *Medicago sativa* (alfalfa) and CDK B1 from *Dunaliella tertiolecta*. Further preferably the class 1 B-type CDK is a CDK B1;1 from *Arabidopsis thaliana* or a CDK B1;2 from *Arabidopsis thaliana*. Alternatively, the nucleic acid
- 15 is preferably a class 2 B-type CDK, such as a class 2 B-type CDK selected from the examples shown in Fig 1, namely, a CDK B2;1 from *Arabidopsis thaliana*, a CDK B2;2 from *Arabidopsis thaliana*, a CDK B2;1 from *Antirrhinum majus*, a CDK B2;1 from *Mesembryanthemum crassifolium*, a CDK B2;1 from *Medicago sativa*, a CDK B2;1 from *Lycopersicon esculentum* and a CDK B 1 from *Oryza sativa*. Further preferably the class 2 B-type CDK is a CDK B2;2
- 20 from *Arabidopsis thaliana*.

- Most preferably the CDK B1;1 nucleic acid is as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;1 protein is as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment
- 25 thereof. Most preferably the CDK B1;2 nucleic acid is as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;2 protein is as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof. Most preferably the CDK B2;2 nucleic acid is as represented by SEQ ID NO: 5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and
- 30 wherein the CDK B2;2 protein is as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof. Each of the CDK B1;1, CDKB1;2 and CDK B2;2 nucleic acids/proteins also encompass the variant nucleic acids and amino acids as described hereinbefore.

- 35 Plants are then transformed with a construct or vector comprising the sequence of interest (i.e., a nucleic acid sequence capable of modulating expression of nucleic acid encoding a B-type CDK protein or a nucleic acid encoding a CDK mutant), which sequence is operably

103-CDKB-PROV

linked to one or more control sequences (at least a promoter). The terms "regulatory element", "control sequence" and "promoter" are all used interchangeably herein and are taken to refer to regulatory nucleic acid sequences capable of effecting expression of the sequences to which they are ligated. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or -10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ. The terms "control sequence", "regulatory sequence", "regulatory element" and "promoter" are used interchangeably herein. The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

Advantageously, the nucleic acid sequence capable of modulating expression of a gene encoding a B-type CDK protein may be operably linked to any promoter. Preferably, in the case of a CDK B1;1, expression is driven by a promoter active in young, expanding tissue, such as young leaves, flowers, stems and roots. Such a "young expanding tissue-preferred promoter" as defined herein refers to a promoter that is expressed predominantly in young expanding tissue, but not necessarily exclusively in such tissue. Preferably, the "young expanding tissue-preferred promoter" is the beta-expansin EXPB8 promoter from rice. Other suitable promoters include any expansin promoter, pLEAFY and others. Preferably in the case of a CDK B1;2 and CDK B2;2 expression is driven in a constitutive manner, most preferably wherein the constitutive promoter is a GOS2 promoter. A constitutive promoter is transcriptionally active during most, but not necessarily all, phases of its growth and development. Examples of constitutive plant promoters are given in Table C as shown below. The promoters shown in Table C below may advantageously be used to practise the method according to the invention.

103-CDKB-PROV

Table C: Examples of Constitutive Promoters

Gene Source	Expression Pattern	Reference
Actin	Constitutive	McElroy <i>et al</i> , Plant Cell, 2: 163-171, 1990
CAMV 35S	Constitutive	Odell <i>et al</i> , Nature, 313: 810-812, 1985
CaMV 19S	Constitutive	Nilsson <i>et al.</i> , <i>Physiol. Plant.</i> 100:456-462, 1997
GOS2	Constitutive	de Pater <i>et al</i> , Plant J Nov;2(6):837-44, 1992
Ubiquitin	Constitutive	Christensen <i>et al</i> , Plant Mol. Biol. 18: 675-689, 1992
Rice cyclophilin	Constitutive	Buchholz <i>et al</i> , Plant Mol Biol. 25(5): 837-43, 1994
Maize H3 histone	Constitutive	Lepetit <i>et al</i> , Mol. Gen. Genet. 231:276-285, 1992
Actin 2	Constitutive	An <i>et al</i> , Plant J. 10(1); 107-121, 1996

Inducible promoters are promoters that have induced or increased transcription initiation in response to a developmental, chemical, environmental or physical stimulus. For example stress-inducible promoters are activated when a plant is exposed to various stress conditions. Examples of stress-inducible promoters, which are also suitable to practise the methods according to the invention, are given in Table D as shown below. Such promoters may also be useful in practising the methods of the invention since modified growth (such as increased growth) induced in times of stress may have many advantages.

10

Table D: Examples of Stress-Inducible Promoters

Name	Stress	Reference
P5CS (delta(1)-pyrroline-5-carboxylate synthase)	Salt, water	Zhang <i>et al</i> ; Plant Science. Oct 28 1997; 129(1): 81-89
cor15a	Cold	Hajela <i>et al.</i> , Plant Physiol. 93: 1246-1252 (1990)
cor15b	Cold	Wihelm <i>et al.</i> , Plant Mol Biol. 1993 Dec; 23(5):1073-7
cor15a (-305 to +78 nt)	Cold, drought	Baker <i>et al.</i> , Plant Mol Biol. 1994 Mar; 24(5): 701-13
rd29	Salt, drought, cold	Kasuga <i>et al.</i> , Nature Biotechnology, vol 18, 287-291, 1999
Heat shock proteins, including artificial promoters containing the heat shock element (HSE)	Heat	Barros <i>et al.</i> , Plant Mol Biol, 19(4): 665-75, 1992. Marrs <i>et al.</i> , Dev Genet., 14(1): 27-41, 1993. Schoffl <i>et al.</i> , Mol Gen Genet, 217(2-3): 246-53, 1989.

103-CDKB-PROV

smHSP (small heat shock proteins)	Heat	Waters et al, J Experimental Botany, vol 47, 296, 325-338, 1996
wcs120	Cold	Ouellet et al., FEBS Lett. 423, 324-328 (1998)
cl7	Cold	Kirch et al., Plant Mol Biol, 33(5): 897-909, 1997 Mar
Adh	Cold, drought, hypoxia	Dolferus et al., Plant Physiol, 105(4): 1075-87, 1994 Aug
pws18	Water: salt and drought	Joshee et al., Plant Cell Physiol, 39(1): 64-72, 1998, Jan
ci21A	Cold	Schneider et al., Plant Physiol, 113(2): 335-45, 1997
Trg-31	Drought	Chaudhary et al., Plant Mol Biol, 30(6): 1247-57, 1996
Osmotin	Osmotic	Raghothama et al., Plant Mol Biol, 23(6): 1117-28, 1993
LapA	Wounding, environmental	WO99/03977 University of California/INRA

The promoters listed in Tables C and D are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention. The promoters listed may also be modified to provide specificity of expression as required.

Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence at the end of a transcriptional unit which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type. One example is when a genetic construct is required to be maintained in a bacterial cell as an episomal genetic

103-CDKB-PROV

element (e.g. plasmid or cosmid molecule). Preferred origins of replication include, but are not limited to, the f1-ori and colE1.

5 The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a nucleic acid construct of the invention. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that
10 kill untransformed cells. Examples of selectable marker genes include the bar gene which provides resistance to the herbicide Basta; the npt gene which confers resistance to the antibiotic kanamycin; the hpt gene which confers hygromycin resistance. Visual markers, such as the Green Fluorescent Protein (GFP, Haseloff et al., 1997), β -glucuronidase (GUS) or luciferase may also be used as selectable markers. Further examples of suitable selectable
15 marker genes include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tet^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (nptII), hygromycin resistance gene, gene, and the chloramphenicol acetyltransferase (CAT) gene, amongst others.

20 The present invention also encompasses plants obtainable by the methods according to the present invention. The present invention therefore provides plants obtainable by the methods according to the present invention, which plants have modified growth characteristics and which plants have altered B-type CDK protein activity and/or levels and/or altered expression of a B-type CDK nucleic acid. These plants may have been obtained by any of the methods
25 described hereinbefore (for example as a result of modulation of B-type CDK levels using recombinant means and/or chemical means) or using any other method.

The present invention also provides plants having modified growth characteristics, which plants have altered B-type CDK protein activity and/or levels and/or altered expression of a B-
30 type CDK nucleic acid.

According to a sixth embodiment of the present invention, there is provided a method for the production of transgenic plants having modified growth characteristics, comprising introduction and expression in a plant of a nucleic acid molecule of the invention.
35

More specifically, the present invention provides a method for the production of transgenic plants having modified growth characteristics, which method comprises:

103-CDKB-PROV

- (i) Introducing into a plant or a plant cell a nucleic acid capable of modulating expression of a B-type CDK gene/nucleic acid and/or activity and/or levels of a B-type CDK protein; or
- (ii) a nucleic acid encoding a CDK mutant, which CDK mutant comprises at least one of the seven amino acid position changes shown in Table A;
- (iii) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

The nucleic acid itself may be introduced directly into a plant cell or into the plant itself (including introduction into a tissue, organ or any other part of the plant). According to a preferred feature of the present invention, the nucleic acid is preferably introduced into a plant by transformation.

The nucleic acid encoding a CDK mutant may be any of the mutant-encoding nucleic acids described hereinbefore.

The nucleic acid capable of modulating expression of a nucleic acid encoding a B-type CDK protein and/or activity of the B-type CDK protein itself may be any of the nucleic acids hereinbefore described. Preferably, the nucleic acid is a B-type CDK nucleic acid/gene. The nucleic acid is preferably a class 1 B-type CDK, such as a class 1 B-type CDK selected from the examples of class 1 CDKs shown in Fig. 1, namely, CDK B1;1 from *Arabidopsis thaliana*, CDK B1;2 from *Arabidopsis thaliana*, CDKB1;1 from *Lycopersicon esculentum* (tomato), CDK B1;1 from *Antirrhinum majus*, CDK B1;1 from *Medicago sativa* (alfalfa) and CDK B1 from *Dunaliella tertiolecta*. Further preferably the class 1 B-type CDK is a CDK B1;1 from *Arabidopsis thaliana* or a CDK B1;2 from *Arabidopsis thaliana*. Alternatively, the nucleic acid is preferably a class 2 B-type CDK, such as a class 2 B-type CDK selected from the examples shown in Fig 1, namely, a CDK B2;1 from *Arabidopsis thaliana*, a CDK B2;2 from *Arabidopsis thaliana*, a CDK B2;1 from *Antirrhinum majus*, a CDK B2;1 from *Mesembryanthemum crassifolium*, a CDK B2;1 from *Medicago sativa*, a CDK B2;1 from *Lycopersicon esculentum* and a CDK B 1 from *Oryza sativa*. Further preferably the class 2 B-type CDK is a CDK B2;2 from *Arabidopsis thaliana*.

Most preferably the CDK B1;1 nucleic acid is as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;1 protein is as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment thereof. Most preferably the CDK B1;2 nucleic acid is as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein

103-CDKB-PROV

the CDK B1;2 protein is as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof. Most preferably the CDK B2;2 nucleic acid is as represented by SEQ ID NO: 5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B2;2 protein is as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof. Each of the CDK B1;1, CDKB1;2 and CDK B2;2 nucleic acids/proteins also encompass the variant nucleic acids and amino acids as described hereinbefore.

The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known to persons skilled in the art.

Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1982, *Nature* 296, 72-74; Negrotti I. et al., June 1987, *Plant Mol. Biol.* 8, 363-373); electroporation of protoplasts (Shillito R.D. et al., 1985 *Bio/Technol* 3, 1099-1102); microinjection into plant material (Crossway A. et al., 1986, *Mol. Gen. Genet* 202, 179-185); DNA or RNA-coated particle bombardment (Klein T.M. et al., 1987, *Nature* 327, 70) infection with (non-integrative) viruses and the like. Transgenic rice plants expressing a B-type CDK gene are preferably produced via *Agrobacterium*-mediated transformation using any of the well known methods for rice transformation, such as described in any of the following: published European patent application EP 1198985 A1, Aldemita and Hodges (*Planta*, 199, 612-617, 1996); Chan et al. (*Plant Mol. Biol.* 22 (3) 491-506, 1993), Hiei et al. (*Plant J.* 6 (2) 271-282,

103-CDKB-PROV

1994), which disclosures are incorporated by reference herein as if fully set forth. In the case of corn transformation, the preferred method is as described in either Ishida *et al.* (Nat. Biotechnol. 1996 Jun; 14(6): 745-50) or Frame *et al.* (Plant Physiol. 2002 May; 129(1): 13-22), which disclosures are incorporated by reference herein as if fully set forth.

5

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant.

10 Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be monitored using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

15

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

20

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

25

The present invention clearly extends to any plant cell or plant produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention. The invention also includes host cells containing an isolated nucleic acid molecule encoding a protein capable of modulating a B-type CDK protein, preferably wherein the protein is a B-type CDK protein. Preferred host cells according to the invention are plant cells. The invention also extends to harvestable parts of a plant, such as but not limited to, seeds, leaves, fruits, flowers, stem cultures, rhizomes, tubers and bulbs.

30

35

103-CDKB-PROV

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include algae, ferns, and all plants which belong to the superfamily *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants, including a fodder or forage legumes, ornamental plants, food crops, trees, or shrubs selected from the list comprising *Abelmoschus* spp., *Acer* spp., *Actinidia* spp., *Agropyron* spp., *Allium* spp., *Amaranthus* spp., *Ananas comosus*, *Annona* spp., *Apium graveolens*, *Arabidopsis thaliana*, *Arachis* spp., *Artocarpus* spp., *Asparagus officinalis*, *Avena sativa*, *Averrhoa carambola*, *Benincasa hispida*, *Bertholletia excelsa*, *Beta vulgaris*, *Brassica* spp., *Cadaba farinosa*, *Camellia sinensis*, *Canna indica*, *Capsicum* spp., *Carica papaya*, *Carissa macrocarpa*, *Carthamus tinctorius*, *Carya* spp., *Castanea* spp., *Cichorium endivia*, *Cinnamomum* spp., *Citrullus lanatus*, *Citrus* spp., *Cocos* spp., *Coffea* spp., *Cola* spp., *Colocasia esculenta*, *Corylus* spp., *Crataegus* spp., *Cucumis* spp., *Cucurbita* spp., *Cynara* spp., *Daucus carota*, *Desmodium* spp., *Dimocarpus longan*, *Dioscorea* spp., *Diospyros* spp., *Echinochloa* spp., *Eleusine coracana*, *Eriobotrya japonica*, *Eugenia uniflora*, *Fagopyrum* spp., *Fagus* spp., *Ficus carica*, *Fortunella* spp., *Fragaria* spp., *Ginkgo biloba*, *Glycine* spp., *Gossypium hirsutum*, *Helianthus* spp., *Hibiscus* spp., *Hordeum* spp., *Ipomoea batatas*, *Juglans* spp., *Lactuca sativa*, *Lathyrus* spp., *Lemna* spp., *Lens culinaris*, *Linum usitatissimum*, *Litchi chinensis*, *Lotus* spp., *Luffa acutangula*, *Lupinus* spp., *Macrotyloma* spp., *Malpighia emarginata*, *Malus* spp., *Mammea americana*, *Mangifera indica*, *Manihot* spp., *Manilkara zapota*, *Medicago sativa*, *Melilotus* spp., *Mentha* spp., *Momordica* spp., *Morus nigra*, *Musa* spp., *Nicotiana* spp., *Olea* spp., *Opuntia* spp., *Ornithopus* spp., *Oryza* spp., *Panicum miliaceum*, *Passiflora edulis*, *Pastinaca sativa*, *Persea* spp., *Petroselinum crispum*, *Phaseolus* spp., *Phoenix* spp., *Physalis* spp., *Pinus* spp., *Pistacia vera*, *Pisum* spp., *Poa* spp., *Populus* spp., *Prosopis* spp., *Prunus* spp., *Psidium* spp., *Punica granatum*, *Pyrus communis*, *Quercus* spp., *Raphanus sativus*, *Rheum rhabarbarum*, *Ribes* spp., *Rubus* spp., *Saccharum* spp., *Sambucus* spp., *Secale cereale*, *Sesamum* spp., *Solanum* spp., *Sorghum bicolor*, *Spinacia* spp., *Syzygium* spp., *Tamarindus indica*, *Theobroma cacao*, *Trifolium* spp., *Triticosecale rimpaul*, *Triticum* spp., *Vaccinium* spp., *Vicia* spp., *Vigna* spp., *Vitis* spp., *Zea mays*, *Zizania palustris*, *Ziziphus* spp., amongst others.

According to a preferred feature of the present invention, the plant is a crop plant, such as soybean, sunflower, canola, alfalfa, rapeseed or cotton. Further preferably, the plant according to the present invention is a monocotyledonous plant, such as sugarcane, most preferably a cereal, such as rice, maize, wheat, millet and barley.

103-CDKB-PROV

Advantageously, performance of the methods according to the present invention results in plants having a variety of modified growth characteristics, such modified growth characteristics including altered cell cycle, increased yield, increased growth rate, modified stress response and modified architecture, each relative to corresponding wild type plants.

The term "altered cell cycle" as used herein encompasses, but is not limited to, an altered (increased or decreased) rate of completion of the cell cycle (i.e., progression through the sequential phases G1 – S – G2 – M) relative to corresponding wild-type plants, or a faster rate of completion through one or more, or part of one or more, of the different phases of the cell cycle relative to corresponding wild-type plants. Normally, these four phases occur sequentially, however, also encompassed is a modified cell cycle in which one or more of the phases are substantially absent possibly resulting in phenomena such as endomitosis, acytokinesis, polyploidy, polyteny and endoreduplication. Since the transition between the different phases of the cell cycle, and therefore progression through the cell cycle, is driven by CDKs, modifying availability of CDKs will have an effect on how cell cycle proceeds, in both its duration and its total number of cycles before differentiating. This may have immediate effect on both cell number and ratio of differentiated versus undifferentiated cells in a delimited area (meristem size, for example).

The term "increased yield" as defined herein encompasses an increase in biomass (weight) in one or more parts of a plant relative to the biomass of corresponding wild-type plants. The term also encompasses an increase in seed yield, which includes an increase in the biomass of the seed (seed weight) and/or an increase in the number of (filled) seeds and/or in the size of the seeds and/or an increase in seed volume, each relative to corresponding wild-type plants. An increase in seed size and/or volume may also influence the composition of seeds. An increase in seed yield could be due to an increase in the number and/or size of flowers. An increase in yield might also increase the harvest index, which is expressed as a ratio of the total biomass over the yield of harvestable parts, such as seeds.

According to a preferred feature of the present invention, performance of the methods of the invention result in plants having modified yield. Preferably, the modified yield includes at least one of: an increase in area, an increase in the number of panicles, an increase in height, an increase in the number of seeds, an increase in the number of filled seeds, an increase in the total weight of seeds, an increase in thousand kernel weight (TKW) and an increase in harvest index, each relative to control plants. Therefore, according to the present invention, there is provided a method for increasing yield of plants, which method comprises modulating

103-CDKB-PROV

expression in a plant of a B-type CDK and/or modulating activity and/or levels in a plant of a B-type CDK protein.

- 5 Since the transgenic plants according to the present invention have increased yield, it is apparent that these plants exhibit an increased growth rate relative to the growth rate of corresponding wild type plants. The increased growth rate may be in one or more parts of a plant (including seeds), or throughout the whole plant. The increase in growth rate may take place at one or more stages in the life cycle of a plant or during the whole plant life cycle. Increased growth rate during the early stages in the life cycle of a plant may give rise to enhanced vigour. The increase in growth rate may alter the harvest time of a plant allowing 10 plants to be harvested sooner than would otherwise be possible. If the growth rate is sufficiently increased, it may even give rise to the possibility of sowing further seeds of the same plant species (for example sowing and harvesting of rice plants followed by sowing and harvesting of further rice plants all within one conventional growing period) or of different plants 15 species (for example the sowing and harvesting of rice plants followed by, for example, the sowing and optional harvesting of soy bean, potatoes or any other suitable plant), thereby increasing the annual biomass production per acre (due to an increase in the number of times (say in a year) that any particular plant may be grown and harvested). The faster rate of growth may be determined by deriving various parameters from growth curves derived from 20 growth experiments, such parameters may be: T-Mid (the time taken for plants to reach 50% of their maximal size) and T-90 (time taken for plants to reach 90% of their maximal size).

- According to a preferred feature of the present invention, performance of the methods of the invention result in plants having modified growth rate. Therefore, according to the present 25 invention, there is provided a method for increasing the growth rate of plants, which method comprises modulating expression in a plant of a B-type CDK and/or modulating activity and/or levels in a plant of a B-type CDK protein. An increase in growth rate is demonstrated in the examples by the reduced time taken for transgenic plants to reach maturity than control plants.

- 30 An increase in yield also encompasses a better performance of the plant under non-stress conditions as well as under stress conditions compared to wild-type plants. Plants typically respond to exposure to stress by growing more slowly. However, since the transgenic plants according to the present invention have increased yield and increased growth rate, it is apparent that transgenic plants will also grow faster during stress conditions than 35 corresponding wild type plants also exposed to the same stress conditions. The stress conditions will typically be the everyday biotic and/or abiotic (environmental) stresses to which a plant may be exposed. Typical abiotic or environmental stresses include temperature

103-CDKB-PROV

stresses caused by atypical hot or cold/freezing temperatures; salt stress; water stress (drought or excess water). Abiotic stresses may also be caused by chemicals. Biotic stresses as typically those stresses caused by pathogens, such as bacteria, viruses, fungi and insects.

- 5 "Modified architecture" as used herein encompasses the appearance or morphology of a plant, including any one or more structural features or combination of structural features. Such structural features include the shape, size, number, position, texture, arrangement and pattern of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, stem or tiller, petiole, trichome, flower, inflorescence (for monocotyledonous and
10 dicotyledonous plants), panicles, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve elements, phloem or vascular tissue, amongst others. Modified architecture therefore includes all aspects of modified growth of the plant. Plants may also modify their architecture in response to certain conditions, such as stress and pathogens (e.g.
15 fungi). Therefore, within the scope of the term "architecture" is included modified architecture under conditions such as stress (or simply sub-optimal conditions) and pathogens.

- According to a preferred feature of the present invention, performance of the methods according to the present invention result in plants having modified architecture. Preferably, the
20 modified architecture includes at least one of: an increase in aboveground area, an increase in the number of panicles and an increase in height. Therefore, according to the present invention, there is provided a method for modifying the architecture of plants, comprising modulating expression in a plant of a B-type CDK nucleic acid/gene and/or modulating activity and/or levels in a plant of a B-type CDK protein.

- 25 The methods according to the present invention result in plants having modified growth characteristics, as described hereinbefore. These advantageous growth characteristics may be combined with other economically advantageous traits, such as further yield-enhancing traits, tolerance to various stresses, traits modifying various architectural features and/or
30 biochemical and/or physiological features.

- According to a further embodiment of the present invention, the use of a B-type CDK is provided. For example, B-type CDKs may be used in breeding programmes. The nucleic acid sequence may be on a chromosome, or a part thereof, comprising at least the nucleic acid
35 sequence encoding the B-type CDK protein and preferably also one or more related family members. In an example of such a breeding programme, a DNA marker is identified which may be genetically linked to a gene capable of modulating expression of a nucleic acid

103-CDKB-PROV

encoding a B-type CDK protein in a plant, which gene may be a gene encoding the B-type CDK protein itself or any other gene capable of directly or indirectly influencing expression of a B-type CDK gene and/or activity and/or levels of a B-type CDK protein. This DNA marker may then used in breeding programs to select plants having altered growth characteristics.

5

Allelic variants of B-type CDKs may be used in particular conventional breeding programmes, such as in marker-assisted breeding. Such breeding programmes sometimes require the introduction of allelic variations in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give rise to altered growth characteristics of a plant. Selection is typically carried out by monitoring growth performance of plants containing different allelic variants of the sequence in question, for example, different allelic variants of SEQ ID NO: 1. Monitoring growth performance can be done in a greenhouse or in the field. Further optional steps include crossing plants, in which the superior allelic variant was identified, with another plant. This could be used, for example, to make a combination of interesting phenotypic features. Allelic variants also encompass Single Nucleotide Polymorphisms (SNPs), as well as Small Insertion/Deletion Polymorphisms (INDELs). The size of INDELs is usually less than 100 bp). SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms.

The present invention also relates to use of a B-type CDK nucleic acid/gene and to use of a B-type CDK protein in modifying the growth characteristics of plants, preferably in modifying one or more of the following characteristics: increasing the area of a plant, increasing the number of first panicles, increasing plant height, increasing the number of seeds, increasing the number of filled seeds, increasing the total weight of seeds, increasing the growth rate, increasing the harvest index and increasing the thousand kernel weight (TKW).

The B-type CDK nucleic acid may be isolated from a monocotyledonous or dicotyledonous species, preferably from the family *Brassicaceae*, further preferably from *Arabidopsis thaliana*. The nucleic acid is preferably a class 1 B-type CDK, such as a class 1 B-type CDK selected from the examples of class 1 CDKs shown in Fig. 1, namely, CDK B1;1 from *Arabidopsis thaliana*, CDK B1;2 from *Arabidopsis thaliana*, CDKB1;1 from *Lycopersicon esculentum* (tomato), CDK B1;1 from *Antirrhinum majus*, CDK B1;1 from *Medicago sativa* (alfalfa) and CDK B1 from *Dunaliella tertiolecta*. Further preferably the class 1 B-type CDK is a CDK B1;1 from *Arabidopsis thaliana* or a CDK B1;2 from *Arabidopsis thaliana*. Alternatively, the nucleic acid is preferably a class 2 B-type CDK, such as a class 2 B-type CDK selected from the

103-CDKB-PROV

examples shown in Fig 1, namely, a CDK B2;1 from *Arabidopsis thaliana*, a CDK B2;2 from *Arabidopsis thaliana*, a CDK B2;1 from *Antirrhinum majus*, a CDK B2;1 from *Mesembryanthemum crassifolium*, a CDK B2;1 from *Medicago sativa*, a CDK B2;1 from *Lycopersicon esculentum* and a CDK B 1 from *Oryza sativa*. Further preferably the class 2 B-type CDK is a CDK B2;2 from *Arabidopsis thaliana*.

Most preferably the CDK B1;1 nucleic acid is as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;1 protein is as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment thereof. Most preferably the CDK B1;2 nucleic acid is as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;2 protein is as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof. Most preferably the CDK B2;2 nucleic acid is as represented by SEQ ID NO: 5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B2;2 protein is as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof. Each of the CDK B1;1, CDKB1;2 and CDK B2;2 nucleic acids/proteins also encompass the variant nucleic acids and amino acids as described hereinbefore.

The present invention also relates to the use of a B-type CDK nucleic acid/gene and/or to the use of a B-type CDK protein as growth regulators. The B-type CDK nucleic acid sequences hereinbefore described and the B-type CDK amino acid sequences hereinbefore described are clearly useful in modifying the growth characteristics of plants. The sequences would therefore find use as growth regulators or growth stimulators. The present invention also provides a composition comprising a B-type CDK protein as hereinbefore described for the use as a growth regulator.

Conversely, the sequences according to the present invention may also be interesting targets for agrochemical compounds, such as herbicides. Accordingly, the present invention encompasses use of the aforementioned B-type CDK nucleic acids as targets for agrochemical compounds, such as herbicides.

Description of figures

The present invention will now be described with reference to the following figures in which:

Fig. 1 is a phylogenetic tree showing the relationship of CDKs from various plants.

103-CDKB-PROV

Fig. 2 is an enlarged view of the CDK A branch of the phylogenetic tree of Fig. 1.

Fig. 3 is a map of the binary vector for the expression in *Oryza sativa* of an *Arabidopsis thaliana* CDK B1;1 gene under the control of a putative beta-expansin promoter, EXPB8 (SEQ ID NO: 14).

Fig. 4 is a map of the binary vector for the expression in *Oryza sativa* of an *Arabidopsis thaliana* CDK B1;2 gene under the control of a GOS2 promoter (SEQ ID NO: 15).

Fig. 5 is a map of the binary vector for the expression in *Oryza sativa* of an *Arabidopsis thaliana* CDK B2;2 gene under the control of a GOS 2 promoter (SEQ ID NO: 15).

Fig. 6 details examples of sequences useful in performing the methods according to the present invention.

Examples

The present invention will now be described with reference to the following examples, which are by way of illustration alone.

DNA manipulation: unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in (Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York) or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Gene Cloning - CDK B1;1

The *Arabidopsis* CDK B1;1 was amplified by PCR using as a template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb and original number of clones was 1.59×10^7 cfu. Original titer was determined to be 9.6×10^6 cfu/ml and, after first amplification, 6×10^{11} cfu/ml. After plasmid extraction, 200 ng of template was used in a 50 μ l PCR mix. Primers prm0350 (sense, start codon in bold, AttB1 site in italic: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATGGAGAAGTACGAG AAGCTAGA 3') and prm0351 (reverse, complementary, stop codon in bold, AttB2 site in italic: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGAACTGAGACTTGTCAAGG 3'), which

103-CDKB-PROV

include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 930 bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment was recombined *in vivo* with the pDONR201 plasmid to produce, according to Gateway terminology, an "entry clone", p0438. Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

Example 2: Vector Construction - CDK B1;1

The entry clone p0438 was subsequently used in an LR reaction with p3169, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders: a plant selectable marker, a plant screenable marker and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A putative beta-expansin promoter for expression in young expanding tissue is located upstream of this Gateway cassette.

After the LR recombination step, the resulting expression vector as shown in Fig. 3 (CDK B1;1: beta-expansin - overexpression) was transformed into *Agrobacterium* and subsequently to *Oryza sativa* plants. Transformed rice plants were allowed to grow to and then examined for various parameters as described in Example 7.

Example 3: Gene Cloning - CDK B1;2

The *Arabidopsis* CDK B1;2 was amplified by PCR using as a template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb, and original number of clones was of 1.59×10^7 cfu. Original titer was determined to be 9.6×10^5 cfu/ml, after first amplification of 6×10^{11} cfu/ml. After plasmid extraction, 200 ng of template was used in a 50 µl PCR mix. Primers prm439 (sense, start codon in bold, AttB1 site in italic: 5' GGGGACAAGTTTGTACAAAAAGCAGGCTTCACAATGGAGAAATACG AGAAGCTC 3') and prm440 (reverse, complementary, stop codon in bold, AttB2 site in italic: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTGGTCAGAACTGAGATTTGTC 3'), which include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 936 bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment was recombined *in vivo* with the pDONR201 plasmid to produce, according to the Gateway

103-CDKB-PROV

terminology, an "entry clone", p538. Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology

Example 4: Vector Construction CDK B1;2

- 5 The entry clone p538 was subsequently used in an LR reaction with p640, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders: a plant selectable marker; a plant screenable marker; and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A GOS2 promoter for upregulation was located upstream of this Gateway cassette.

10

After the LR recombination step, the resulting expression vector as shown in Fig. 4 (CDK B1;2: GOS 2 - overexpression) was transformed into *Agrobacterium* and subsequently to *Oryza sativa* plants. Transformed rice plants were allowed to grow to and then examined for various parameters as described in Example 7.

15

Example 5: Gene Cloning – CDK B2;2

- The *Arabidopsis* CDKB2;2 was amplified by PCR using as a template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb, and original number of clones was of 1.59×10^7 cfu. Original titer was determined to be 9.6×10^5 cfu/ml, after first amplification of 6×10^{11} cfu/ml. After plasmid extraction, 200 ng of template was used in a 50 µl PCR mix. Primers prm2213 (sense, start codon in bold, AttB1 site in italic: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACA – GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATGGACAACAATGGAGTTAA 3') and prm2214 (reverse, complementary, stop codon in bold, AttB2 site in italic: 5' GGGGACCACTTTGTACAAGAAAGCTGGG – GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGAGAGAGGACTTGTCTAG 3'), which include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 948 bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment was recombined *in vivo* with the pDONR201 plasmid to produce, according to Gateway terminology, an "entry clone", p2660. Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

35 Example 6: Vector Construction – CDK B2;2

The entry clone p2660 was subsequently used in an LR reaction with p640, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within

103-CDKB-PROV

the T-DNA borders: a plant selectable marker; a plant screenable marker; and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A pGOS2 promoter for overexpression was located upstream of this Gateway cassette.

5

After the LR recombination step, the resulting expression vector as shown in Fig. 5 (CDK B2;2: GOS2 - overexpression) was transformed into *Agrobacterium* and subsequently into *Oryza sativa* plants. Transformed rice plants were allowed to grow to and then examined for various parameters as described in Example 7.

10

Example 7: Evaluation and Results

Approximately 15 to 20 independent T0 rice transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. 5 events, of which the T1 progeny segregated 3:1 for presence/absence of the transgene, were retained. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homo-zygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by monitoring visual marker expression.

Statistical analysis: t-test and F-test

20 A two factor ANOVA (analysis of variants) was used as statistical model for the overall evaluation of plant phenotypic characteristics. An F-test was carried out on all the parameters measured, for all of the plants of all of the events transformed with the gene of interest. The F-test was carried out to check for an effect of the gene over all the transformation events and to determine the overall effect of the gene or "global gene effect". Significant data, as determined by the value of the F-test, indicates a "gene" effect, meaning that the phenotype observed is caused by more than the presence or position of the gene. In the case of the F-test, the threshold for significance for a global gene effect is set at a 5% probability level.

30 To check for an effect of the genes within an event, i.e., for a line-specific effect, a t-test was performed within each event using data sets from the transgenic plants and the corresponding null plants. "Null plants" or "Null segregants" are the plants treated in the same way as the transgenic plant, but from which the transgene has segregated. Null plants can also be described as the homozygous negative transformants. The threshold for significance for the t-test is set at 10% probability level. Within one population of transformation events, some events can be under or above this t-test threshold. This is based on the hypothesis that a gene might only have an effect in certain positions in the genome, and that the occurrence of this position-dependent effect is not uncommon. This kind of gene effect may also be referred

103-CDKB-PROV

to as a "line effect of a gene". The p-value is obtained by comparing the t-value to the t-distribution or alternatively, by comparing the F-value to the F-distribution. The p-value stands for the probability that the null hypothesis (null hypothesis being "there is no effect of the transgene") is correct.

5

7.1 Vegetative growth measurements:

The selected T1 plants (approximately 10 with the transgene and approximately 10 without the transgene) were transferred to a greenhouse. Each plant received a unique barcode label to link unambiguously the phenotyping data to the corresponding plant. The selected T1 plants were grown on soil in 10 cm diameter pots under the following environmental settings: photoperiod= 11.5 h, daylight intensity= 30,000 lux or more, daytime temperature= 28°C or higher, night time temperature= 22°C, relative humidity= 60-70%. Transgenic plants and the corresponding nullzygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity each plant was passed several times through a digital imaging cabinet and imaged. At each time point digital images (2048x1536 pixels, 16 million colours) were taken of each plant from at least 6 different angles. The parameters described below were derived in an automated way from all the digital images of all the plants, using image analysis software.

20 (a) Aboveground plant area

Plant aboveground area was determined by counting the total number of pixels from aboveground plant parts discriminated from the background. This value was averaged for the pictures taken on the same time point from the different angles and was converted to a physical surface value expressed in square mm by calibration. Experiments show that the aboveground plant area measured this way correlates with the biomass of plant parts above ground.

Table 1: Aboveground plant area – CDK B1;1

Aboveground plant area – CDK B1;1					
Line	TR	null	dif	% dif	p-value
1	58568	60355	-1787	-3	0.7936
2	56252	50784	5467	11	0.4897
3	76774	50047	26727	53	0.0011
4	63535	57864	5671	10	0.4096
5	66544	68217	-1673	-2	0.8065
Overall	64192	58360	5832	10	0.0729

103-CDKB-PROV

Each row corresponds to one event, for which the aboveground area was determined for the transgenic plants (TR) and the null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

As shown in Table 1, line 3 gave a significant increase in the aboveground area for transgenic plants relative to control plants, with a p-value from the t-test of 0.0011. Lines 2 and 4 also showed an increase in aboveground plant area relative to that of control plants. An overall increase of 10% was seen in the aboveground area of transgenic plants compared to control plants.

Table 2: Aboveground plant area – CDK B1;2

Above ground plant area – CDK B1;2					
Line	TR	null	dif	% dif	p-value
10	32651	34319	-1667	-5	0.6726
11	58946	43829	15117	34	0.0002
12	46916	39524	7392	19	0.0573
13	48268	51538	-3269	-6	0.4359
Overall	46724	42099	4625	11	0.0178

Each row corresponds to one event, for which the aboveground area was determined for the transgenic plants (TR) and the null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

As shown in Table 2, lines 11 and 12 gave a significant increase in aboveground plant area with respective p-values from the t-test of 0.0002 and 0.0573. An overall gene effect was also apparent from a p-value of 0.0178 from the F-test. An overall increase of 11% was seen in the aboveground area of transgenic plants compared to control plants.

(b) Plant height

Plant height was determined by the distance between the horizontal lines going through the upper pot edge and the uppermost pixel corresponding to a plant part above ground. This value was averaged for the pictures taken on the same time point from the different angles and

103-CDKB-PROV

was converted, by calibration, to a physical distance expressed in mm. Experiments showed that plant height measured this way correlates with plant height measured manually with a ruler.

5 **Table 3: Height – CDK B1;1**

Height – CDK B1;1					
Line	TR	null	dif	% dif	p-value
1	902	950	-48	-5	0.3468
2	962	948	13	1	0.8181
3	1055	884	171	19	0.0045
4	913	884	49	6	0.3358
5	970	990	-20	-2	0.7003
Overall	956	929	26	3	0.2693

Each row corresponds to one event, for which plant height was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

The results are shown in Table 3. As shown, line 3 showed a significant increase in plant height relative to corresponding control plants (with a p value from the t-test of 0.0045).

15 **Table 4: Height – CDK B1;2**

Height – CDK B1;2					
Line	TR	null	dif	% dif	p-value
11	1178	1081	96	9	0.019
12	976	1004	-28	-3	0.4815
13	1049	1001	48	5	0.2379
Overall	1068	1029	38	4	0.0945

Each row corresponds to one event, for which plant height was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

103-CDKB-PROV

As shown in Table 4, line 11 showed a significant increase in height relative to control plants with a p value from the t-test of 0.019.

7.2 Seed-related parameter measurements

- 5 The mature primary panicles were harvested, bagged, barcode-labelled and then dried for three days in the oven at 37°C. The panicles were then threshed and all the seeds were collected and counted. The filled husks were separated from the empty ones using an air-blowing device. The empty husks were discarded and the remaining fraction was counted again. The filled husks were weighed on an analytical balance. This procedure resulted in the set of seed-related parameters described below.

10 (c) Total seed number per plant

This was measured by counting the number of husks harvested from a plant.

Table 5: Total seed number – CDK B1;1

Total Seed Number- CDK B1;1					
Line	TR	null	Dif	% dif	p-value
1	563.6	514.2	49.36	10	0.4842
2	380	346	34	10	0.6685
3	729.6	405.2	324.46	80	0.0002
4	476.3	463.5	12.77	3	0.8577
5	506.1	561.8	-55.7	-10	0.4176
Overall	528.1	470.2	57.84	12	0.081

- 15 Each row corresponds to one event, for which the total seed number was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.
- 20

The results are shown in Table 5 above. As shown, line 3 gave a significant increase in the total number of seeds produced by transgenic plants relative to the total number of seeds produced by control plants (with a p value from the t-test of 0.0002).

103-CDKB-PROV

Table 6: Total seed number – CDK B1;2

Total Seed Number – CDK B1;2					
Line	TR	null	dif	% dif	p-value
11	638.7	484.2	154.43	32	0.0047
12	428.4	507.3	-78.88	-16	0.1356
13	425.6	426.6	-0.98	-0	0.9856
Overall	498.2	474.2	24.03	5	0.4425

Each row corresponds to one event, for which the total seed number was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

- 10 As shown in Table 6, line 11 showed a significant increase (with a p value from the t-test of 0.0047) in the total number of seeds of transgenic plants relative to the total number of seeds of control plants.

(d) Number of filled seeds

- 15 The number of filled seeds was determined by counting the number of filled husks that remained after the separation step.

Table 7: Number of filled seeds – CDK B1;1

Number of Filled Seeds – CDK B1;1					
Line	TR	null	dif	% dif	p-value
1	268.4	224.5	43.94	20	0.3353
2	193.5	185.5	8	4	0.881
3	365.4	218.2	147.21	67	0.0072
4	209.3	231.3	-21.98	-10	0.6329
5	292.3	303.2	-10.9	-4	0.8055
Overall	264.6	237.3	27.3	12	0.2026

- 20 Each row corresponds to one event, for which the total number of filled seeds was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.
- 25

103-CDKB-PROV

The results are shown in Table 7 above. As shown, line 3 showed a significant increase in the number of filled seeds relative to that of control plants (with a p value of the t test of 0.0072).

5 Table 8: Number of filled seeds – CDK B1;2

Number of filled seeds – CDK B1;2					
Line	TR	null	dif	% dif	p-value
10	27.5	25	2.5	10	0.7578
11	516.3	330.2	186.05	56	0.0091
12	225.7	275.4	-49.74	-18	0.3294
13	261.3	279.3	-18.05	-6	0.7196
Overall	273.3	239.5	33.83	14	0.3805

Each row corresponds to one event, for which the total number of filled seeds was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

As shown in Table 8, line 11 showed an increase in the number of filled seeds relative to control plants with a p value from the t-test of 0.0091. An overall difference of 14% was observed for the number of filled seeds of transgenic plants relative to the number of filled seeds for corresponding control plants.

(e) Total seed yield per plant

The total seed yield was measured by weighing all filled husks harvested from a plant.

20 Table 9: Total weight of seeds – CDK B1;1

Total Weight Seeds – CDK B1;1					
Line	TR	null	dif	% dif	p-value
1	5.4	4.4	0.94	21	0.3578
2	4.2	3.3	0.93	28	0.4186
3	8	4.6	3.44	75	0.005
4	4.6	5	-0.46	-9	0.6551
5	6.4	6.7	-0.23	-3	0.8182
Overall	5.7	4.9	0.77	16	0.1096

Each row corresponds to one event, for which the total weight of seeds was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage

103-CDKB-PROV

difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

- 5 The results are shown in Table 9 above. As shown, line 3 showed a significant increase (with a p value from the t test of 0.005) in the total weight of seeds of transgenic plants relative to the total weight of the seeds of corresponding non-transgenic plants. An overall increase of 16% was observed for the total weight of the seeds of transgenic plants verses the total weight of the seeds of control plants.

10

Table 10: Total weight of seeds – CDK B1;2

Total Weight Seeds – CDK B1;2					
Line	TR	null	dif	% dif	p-value
10	0.6	0.6	0.01	2	0.7587
11	11.3	7.7	3.55	46	0.0139
12	6	6.8	-0.88	-13	0.4276
13	7	7.5	-0.5	-7	0.6736
Overall	6.3	5.7	0.6	11	0.5841

- Each row corresponds to one event, for which the total weight of seeds was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

- 20 As shown in Table 10, line 11 showed a significant increase in the total weight of the seeds of transgenic plants relative to the total weight of seeds of control plants with a p value from the t-test of 0.0139.

(f) Harvest index of plants

- 25 The harvest index in the present invention is defined as the ratio between the total seed yield and the above ground area (mm²), multiplied by a factor 106.

103-CDKB-PROV

Table 11: Harvest Index – CDK B1;1

Harvest Index – CDK B1;1					
Line	TR	null	dif	% dif	p-value
1	82.3	74.7	7.62	10	0.5097
2	72.7	74.4	-1.75	-2	0.8972
3	103.9	71.8	32.11	45	0.0201
4	87.9	75.8	-7.87	-10	0.5009
5	97.3	97.1	0.19	0	0.9868
Overall	84.4	79.5	4.94	6	0.3644

Each row corresponds to one event, for which the harvest index was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row shows average numbers for all events. In this row, the p-value indicated stands for the p-value from the F-test.

10

The results are shown in Table 11 above. As shown, line 3 showed an increased harvest index for transgenic plants relative to the harvest index of control plants (with a p value from the t-test of 0.0201).

15 (g) Thousand Kernel Weight

Thousand Kernel Weight (TKW): this parameter is extrapolated from the number of filled seeds counted and their total weight.

Table 12: Thousand Kernel Weight (TKW) – CDK B1;2

TKW – CDK B1;2					
Line	TR	null	dif	% dif	p-value
11	22.2	19.5	2.67	14	0.0347
12	22.2	22	0.18	1	0.8554
13	21.2	22.4	-1.2	-5	0.2525
Overall	21.9	21.3	0.55	3	0.4471

20

Each row corresponds to one event, for which the TKW was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant

103-CDKB-PROV

line. The last row shows average numbers for all events. In this row, the *p*-value indicated stands for the *p*-value from the *F*-test.

The results are shown in Table 12 above. As shown, Line 11 gave a significant increase in the TKW of transgenic plants relative to the TKW of control plants with a *p* value from the *t*-test of 0.0347.

(h) Cycle time – CDK B2;2

Weekly plant area measurements were modelled to obtain a growth curve for each plant.

Plant area (in mm²) was plotted against time (in days) and from the resultant growth curve the following parameters were calculated.

Table 13: Growth rate – CDK B2;2

Growth Rate (Total Area Cycle Time) – CDK B2;2					
Line	TR	null	dif	% dif	p-value
20	72	74	-2	-3	0.2143
21	74	77	-3	-4	0.0749
22	70	72	-2	-3	0.2296
23	75	72	3	4	0.0582
24	75	74	1	1	0.4851
25	74	74	-0	-0	0.9693
26	76	77	-1	-1	0.5318
27	71	74	-3	-4	0.0255
Overall	73	74	-1	-1	0.1062

Each row corresponds to one event, for which the growth rate was determined for transgenic plants (TR) and null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage difference between these plants (% dif). *P*-value stands for the probability produced by the *t*-test for each plant line. The last row shows average numbers for all events. In this row, the *p*-value indicated stands for the *p*-value from the *F*-test.

The results are shown in Table 13 above. As shown, lines 21 and 27 gave significant increases in the growth rate of transgenic plants relative to the growth rate of control plants, with increases of 4 days observed in both cases.

103-CDKB-PROV**Example 8: Transgenic Corn Expressing a B-type CDK**

A B type CDK is cloned under control of a young expanding tissue-preferred or a constitutive promoter in a plant transformation vector suitable for *Agrobacterium*-mediated corn transformation. Vectors and methods for corn transformation are selected from those described in any of: EP0604662, EP0672752, EP0971578, EP0955371, EP0568676, Ishida *et al.* (Nat. Biotechnol. 1996 Jun; 14(6): 745-50); and Frame *et al.* (Plant Physiol. 2002 May; 129(1): 13-22).

Transgenic plants made by these methods are grown in the greenhouse for T1 seed production. Inheritability and copy number of the transgene is checked by quantitative real-time PCR and Southern blot analysis. Expression levels of the transgene are determined by reverse PCR and Northern analysis. Transgenic lines with single copy insertions of the transgene and with varying levels of transgene expression are selected for T2 seed production.

Progeny seeds are germinated and grown in a greenhouse in conditions adapted for maize (16:8 photoperiod, 26-28°C daytime temperature and 22-24°C night time temperature) as well under water-deficient, nitrogen-deficient, and excess NaCl conditions. In the case of selfing, null segregants from the same parental line, as well as wild type plants of the same cultivar are used as controls. The progeny plants resulting from the selfing or the crosses are evaluated for different biomass and growth parameters, including plant height, stem thickness, number of leaves, total above ground area, leaf greenness, time to maturity, flowering time, ear number, harvesting time. The seeds of these lines are also evaluated for changes in various parameters, such as grain size, total grain yield per plant, and grain quality (starch content, protein content and oil content).

Lines that are most significantly improved compared to corresponding control lines are selected for further field-testing and marker-assisted breeding, with the objective of transferring the field-validated transgenic traits into commercial germplasm. The testing of maize for growth and yield-related parameters in the field is conducted using well-established protocols. Similarly, introgressing specific loci (such as transgene containing loci) from one germplasm into another is also conducted using well-established protocols.

Example 9: Identification of mutant B-type CDKs

All molecular biology experiments were performed following standard procedures. Gateway destination vectors were amplified using *Escherichia coli* DB3.1 strain (F-, gyrA462, endA-, delta (sr1-recA), mcrB, mrr, hsdS20 (rB-, mB-), supE44, ara14, galK2, lacY1, proA2, rpsL20

103-CDKB-PROV

(S_{mr}), xyl5, lambda-, leu, mt1). Other constructs were amplified using *E. coli* DH5-alpha strain (F-, phi80diacZDelta M15, Delta (lacZYA-argF), U169, deoR, recA1, hadR17 (rk-,mk+), gal-, phoA, supE44, Lambda-, thi-1, gyrA96m, relA1).

5 **9.0: Strains and media used**

Saccharomyces cerevisiae CDK mutant strain US102 (MATa; leu2-3; ura3; trp1-1; his3-11; ade2-1; can1-100; cdc28-1N) was used for complementation studies. Yeast cells were cultivated on rich medium (YPG-gal: yeast extract 0.5 % w/v; peptone 0.5 % w/v; galactose 1 % w/v; raffinose 1 % w/v, agar 2 % w/v) or on synthetic medium (YNB: yeast nitrogen base 10 without amino-acids (Difco, Becton Dickinson, Sparks, Madison, USA) 0.7 % w/v, agar 2 % w/v) supplemented with appropriate sugars and the adequate amino-acid drop-out mixture (Clontech, Palo-Alto, California, USA). Competent yeast cells were prepared and used for transformation using the Frozen-EZ Yeast Transformation II kit (ZymoResearch, Orange, California, USA) following the instructions of the manufacturer.

15

9.1: Two-hybrid vector construction

pAD-Gal4 2.1 and pBD-gal4-cam (Stratagene, La Jolla, California, USA) were cut with *Sma* I, dephosphorylated, ligated to the Gateway cassette C (Life Technologies, Invitrogen Ltd, Paisley, UK) and introduced into *Escherichia coli* DB3.1. The orientation of the cassette was 20 checked. The resulting vectors, pGW-AD and pGW-BD, contained the AttR1 site of the gateway cassette fused respectively to the GAL4 AD or BD domains, thus allowing insertion of coding sequences in-frame to the GAL4 AD or BD domains using the Gateway procedure (Life Technologies, Invitrogen Ltd, Paisley, UK).

25 *Oryza sativa* cdc2-1 (CDK A;1) cyclin dependent kinase full-length cDNA (accession number X60374, see also SEQ ID NOs 7 and 8) was amplified using AttB sites containing primers (Cdc2-1-AttB1: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATGGAGCAGTACGAG AAGGAGGAG, cdc2-1-AttB2: GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCTGTCAT TGTACCATCTCAAG). The PCR products were introduced into pDONR201 (Life 30 Technologies, Invitrogen Ltd, Paisley, UK) using the Gateway BP procedure. The resulting entry clones, pDONR-cdc2-1, were used to transfer the cdc2 coding sequences into the pGW-AD and pGW-BD vectors via the gateway LR reaction. The resulting plasmids, pAD-cdc2-1 and pBD-cdc2-1, contained the rice CDK A;1 coding sequences fused to the GAL4 AD or BD domains.

35

Protein-protein Interactions were investigated following a mating 2-hybrid procedure using PJ69-4A (MATa, ura3-52, his3-200, ade2Δ, trp1-901, leu2-3112, gal4Δ, gal80Δ, LYS2::GAL1-

103-CDKB-PROV

HIS3, ade2::GAL2-ADE2, met2::GAL7-lacZ. James *et al.*, 1996) and PJ69-4alpha (MATalpha, ura3-52, his3-200, ade2Δ trp1-901, leu2-3112, gal4Δ, gal80Δ LYS2::GAL1-HIS3, ade2::GAL2-ADE2, met2::GAL7-lacZ. Uetz *et al.* 2000).

5 9.2: Random mutagenesis

Randomly mutated CDK A;1 coding sequences were produced using error-prone PCR. 30ng of pAD-cdc2-1 plasmid were added to a PCR mix (dATP 0.2 mM, dGTP 0.2 mM, dCTP 1mM, dTTP 1 mM, buffer 1X, MnCl₂ 0.5 mM, forward primer (AGGGATGTTTAATACCACTAC) 1mM, reverse primer (GCACAGTTGAAGTGAAGTTGC) 1-mM, Taq polymerase 1 U). The reaction mixture was denatured for 5 minutes at 94°C, 30 cycles of 1 minute denaturing at 94°C, 1 minute annealing at 40 °C, 2 minutes elongation at 72°C, followed by a last elongation of 5 minutes at 72°C. This procedure introduces errors at a rate of 2-3 base substitutions per kilo base pair (Miyazaki and Arnold (1999). J Mol Evol 49:716-720; Shafikhani *et al* (1997). BioTechniques 23:304-310). The PCR fragment was cloned directly by gap-repair cloning (Fusco *et al.* (1999) Yeast 15: 715-720) into pAD vector linearized by *EcoR* I and *Sal* I digestion, using the yeast strain MaV203 (Life technology) containing the pBD-OsICK2 or pBD-OsICK4 plasmid as recipient. CDK A;1 was selected by reverse-two-hybrid methods. The yeast strains were plated on -Leu -Trp selective medium containing 0.2 % (w/v) 5-fluoro-ortotic acid and incubated at 28°C. Yeast colonies able to survive on such a medium were CDK A;1 mutants unable to interact with ICK2 or ICK4.

9.3: Two-hybrid mating procedure

Yeast strains PJ69-4A (MAT-a) and PJ69-4alpha (MAT-alpha) were transformed either with pAD or pBD plasmid containing the coding sequences of interest. Individual strains were plated in strips, either horizontally or vertically, respectively on -Leu or -Trp selective medium and were grown at 28°C for 1-2 days. The yeast strips were transferred onto non-selective YPG medium in such a way that the strips formed a grid, with AD and BD strains mixing at strip intersections. The yeast was incubated for 8 hours at 28°C to allow mating between MAT-a and MAT-alpha strains to occur. The grid was subsequently transferred on -Leu -Trp -His -Ade selective medium and incubated at 28°C for 2 days. Yeast able to grow on such a medium were MAT-a/alpha diploid yeasts containing AD and BD plasmid expressing interacting proteins (James *et al.* (1996) Genetics 144: 1425-1436. Uetz *et al.* (2000) Nature 403 : 623-627).

35 9.4: Yeast expression vectors construction and mutant complementation

Yeast galactose-inducible expression vector pESC-Trp (Stratagene) was cut with *Apa* I and *Sal* I, ligated to the *Apa* I -*Sal* I fragment from pBSK-GWA, which contains the Gateway

103-CDKB-PROV

cassette A cloned at the *EcoR* V site of pBlueScript. The resulting vectors, pE-GW, was introduced into *E. coli* DB3.1, and contained the *AttR1* site of the gateway cassette directly downstream of the *GAL1* promoter. The coding sequences of interest were transferred into the pE-GW vector from the pDONR entry clone via the gateway LR reaction.

- 5 Yeast strain US102 (Loy CJ *et al.* (1999) Mol Cell Biol. 19: 3312-3327) was mutated in the CDK *cdc28*, and is unable to grow at 37°C, but able to grow at 24°C. Transgenic yeast strain containing plasmids for expression of interesting genes were grown on galactose-containing medium, at 37°C. The yeast strains expressing genes able to rescue the *cdc28* mutation are those able to grow at that restrictive temperature.

RESULTS**9.5: Screening**

- 15 Rice CDK A;1 mutant library in pAD vector was generated by error-prone PCR and gap repair cloning in the MaV203 strain as described. The library was screened against rice ICK4 by reverse two-hybrid. About 100 000 mutants were screened, and 79 colonies able to grow on 5-fluoroorotic acid were obtained. Among them, 74 were transferred into PJ69-4A for further analysis by mating two-hybrid. The mutant coding sequences were then sequenced. An average mutagenicity level of 5.6 ± 3.4 substitutions per kilo base pair was observed.

20

9.6: Characterization

- 25 Wild-type rice *cdc2-1* is able to bind strongly to the rice ICK4 and the mouse herpesvirus Cyclin D homologue and less strongly the rice cyclin D3. The capacity of the *cdc2* mutants to bind these 3 proteins was investigated. Their capacity to complement the US102 *cdc28* yeast mutant strain, like the wild type *cdc2* protein, was also investigated.

9.6.1: Mutants that bind to cyclin, but not to ICKs

- 30 Mutants of particular interest were those able to bind cyclins, but not ICK4, whilst still retaining their capacity to complement the yeast mutant. These mutants render a cyclin-CDK complex insensitive to ICK-mediated inhibition. Three mutants of particular interest identified are shown in Table A repeated below.

Table A: Mutants that bind to cyclin, but not to ICKs

Mutant	SEQ ID NO	Mutation Position		
		Y4H	V79D	A152T
1	SEQ ID NO: 9			
2	SEQ ID NO: 10	I30T		
3	SEQ ID NO: 11	E5V	R122S	K143E

103-CDKB-PROV

Mutations positions are calculated from the first methionine of CDK A;1.

- 5 9.6.2: Mutants that bind CKI but not cyclin
Also interesting are those mutants able to bind ICK, but not cyclins, and which have lost their capacity to complement the yeast US102 mutant. Such mutants would titrate out the ICKs rendering them unable to inhibit the CDK-cyclin complex. The mutants shown in Table B repeated below show such characteristics.

10 **Table B: Mutants that bind CKI but not cyclin**

Mutant	SEQ ID NO	Mutant Position						
4	SEQ ID NO: 12	G154R						
5	SEQ ID NO: 13	Q3P	E38A	R137L	S182A	I193T	M267V	R279Q

103-CDKB-PROV

Claims

1. Screening method for the identification of mutant cyclin dependent kinases (CDKs) having enhanced CDK activity relative to corresponding non-mutated CDKs, which method comprises the steps of:
- 5 (a) Providing CDK mutants;
(b) Identifying cyclin dependent kinase inhibitor (ICK) non-reacting mutants;
(c) Identifying mutants having cyclin-binding activity; and optionally followed by,
(d) A yeast complementation assay on resultant mutants from steps (b) and (c).
- 10 2. Screening method for the identification of substantially non-active CDKs, but which are capable of binding to ICKs, comprising the steps of:
- 15 (a) Providing CDK mutants;
(b) Identifying ICK binding mutants; and
(c) Identifying non-cyclin-binding mutants.
3. Screening method according to claim 2 or 3 wherein said CDK mutants are provided by the steps of:
- 20 (a) Providing a wild-type CDK; and
(b) Mutating said CDK at least at 1 amino-acid position;
4. Mutant CDKs obtainable by the method according to claim 1 or 3, which mutant CDKs are indicated in Table A.
- 25 5. Mutant CDKs obtainable by the method according to claim 2 or 3, which mutant CDKs are indicated in Table B.
6. An isolated nucleic acid molecule comprising:
- 30 (a) a nucleic acid encoding a CDK mutant represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13;
(b) a nucleic acid encoding a homologue, derivative or active fragment of a CDK mutant represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, which homologue, derivative or active fragment preferably comprises at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;
- 35 (c) a nucleic acid capable of hybridising with a nucleic acid of (a) or (b) above, wherein the hybridising sequence preferably encodes an amino acid comprising at least one of the

103-CDKB-PROV

seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;

(d) a nucleic acid of (a) to (c) above which is degenerate as a result of the genetic code;

(e) allelic variants of the nucleic acids of (a) to (d), which allelic variant preferably encodes an amino acid comprising at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B; and

(f) alternative splice variants of the nucleic acids of (a) to (e), which alternative splice variants preferably encode an amino acid comprising at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B.

7. A CDK mutant, comprising:

(a) an amino acid represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13; and

(c) a fragment of an amino acid represented by any one of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, which fragment comprises at least seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B.

8. Method for modifying plant growth characteristics, comprising modulating expression in a plant of a nucleic acid encoding a B-type CDK protein and/or modulating activity and/or levels in a plant of a B-type CDK protein.

9. Method according to claim 8, wherein said modulation is effected by recombinant means and/or chemical means.

10. Method according to claim 9, wherein said chemical means comprises the exogenous application of one or more compounds or elements capable of modulating expression in a plant of a B-type CDK nucleic acid and/or modulating activity and/or levels of a B-type CDK protein in a plant.

11. Method according to any one of claims 8 to 10, wherein said modulating expression comprises introducing into a plant a nucleic acid capable of modulating expression in a plant of a B-type CDK nucleic acid and/or modulating activity and/or levels of a B-type CDK protein in a plant.

103-CDKB-PROV

12. Method according to claim 11, wherein said nucleic acid is selected from:

- (a) Nucleic acids encoding transcription factors, activators or inhibitors of a B-type CDK;
- (b) B-type CDK regulatory sequences; and
- (c) Ligands of a B-type CDK protein.

5

13. Method according to claim 11, wherein said nucleic acid is a B-type CDK.

14. Method according to claim 13, wherein said B-type CDK is derived from a microbe, plant, algae or animal.

10

15. Method according to claim 14, wherein said B-type CDK derived from a plant is preferably from a dicotyledonous plant, further preferably from the family *Brassicaceae*, more preferably the nucleic acid sequence is from *Arabidopsis thaliana*.

15

16. Method according to any one of claims 13 to 15, wherein said B-type CDK is a class 1 B-type CDK, preferably a CDK B1;1 from *Arabidopsis thaliana* or a CDK B1;2 from *Arabidopsis thaliana*.

20

17. Method according to any one of claims 13 to 15, wherein said B-type CDK is a class 2 B-type CDK, preferably a CDK B2;2 from *Arabidopsis thaliana*.

25

18. Method according to claim 16, wherein said CDK B1;1 nucleic acid is as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;1 protein is as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment thereof.

30

19. Method according to claim 16, wherein said CDK B1;2 nucleic acid is as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B1;2 protein is as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof.

35

20. Method according to claim 17, wherein said CDK B2;2 nucleic acid is as represented by SEQ ID NO: 5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, and wherein the CDK B2;2 protein is as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof.

103-CDKB-PROV

21. Method according to any of claims 16 to 20, wherein said B-type CDK includes variant nucleic acid and amino acids selected from:

- (a) Functional portions of a B-type CDK nucleic acid/gene;
- (b) Sequences capable of hybridising to a B-type CDK nucleic acid/gene;
- (c) Alternative splice variants of a B-type CDK nucleic acid/gene;
- (d) Allelic variants of a B-type CDK nucleic acid/gene;
- (e) Homologues, derivatives and active fragments of a B-type CDK protein;
- (f) Mutant B-type CDKs;

22. Method according to any one of claims 8 to 21, wherein said B-type CDK protein comprises at least a PPTALRE motif.

23. Method according to any one of claims 8 to 22, wherein said B-type CDK protein comprises at least one of the seven amino acid position changes shown in Table A.

24. Method according to any one of claims 8 to 23, wherein said B-type CDK is overexpressed in a plant.

25. Method according to any one of claims 16 to 24, wherein expression of said CDK B1;1 nucleic acid is driven by a promoter active in young, expanding tissue, preferably wherein said promoter is a beta expansin promoter.

26. Method according to any one of claims 16 to 24, wherein expression of said CDK B1;2 nucleic acid and wherein expression of said CDK B2;2 nucleic acid is driven by a constitutive promoter, preferably wherein said promoter is a GOS 2 promoter.

27. Method according to any of claims 8 to 26, wherein said modified growth characteristic is selected from any one or more of altered cell cycle, increased yield, increased growth rate, modified stress response and modified plant architecture, each relative to corresponding wild type plants.

28. Method according to claim 27, wherein said modified yield comprises one or more of: an increase in area, an increase in the number of panicles, an increase in height, an increase in the number of seeds, an increase in the number of filled seeds, an increase in the total weight of seeds, an increase in thousand kernel weight (TKW) and an increase in harvest index, each relative to control plants.

103-CDKB-PROV

29. Method according to claim 27 or 28, wherein said modified architecture includes one or more of: increase in aboveground area, increase in the number of panicles and an increase in height.

5 30. A method for increasing the yield of plants, which method comprises modulating expression in a plant of a B-type CDK and/or modulating activity and/or levels in a plant of a B-type CDK protein.

10 31. A method for modifying the architecture of plants, comprising modulating expression in a plant of a B-type CDK nucleic acid/gene and/or modulating activity and/or levels in a plant of a B-type CDK protein.

15 32. A method for increasing the growth rate of plants, which method comprises modulating expression in a plant of a B-type CDK and/or modulating activity and/or levels in a plant of a B-type CDK protein.

33. Plants obtainable by a method according to any of claims 8 to 32.

34. Construct comprising:

- 20 (a) a nucleic acid capable of modulating expression of a B-type CDK gene/nucleic acid and/or activity and/or levels of a B-type CDK protein; or
- (b) a nucleic acid encoding a CDK mutant, which CDK mutant comprises at least one of the seven amino acid position changes shown in Table A or at least one of the eight amino acid position changes shown in Table B;
- 25 (c) one or more control sequences capable of driving expression of the nucleic acid of (a) or (b); and optionally
- (d) a transcription termination sequence.

30 35. Construct according to claim 34, wherein said nucleic acid of (a) is a CDK B1;1 nucleic acid as represented by SEQ ID NO: 1 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, which nucleic acid encodes a CDK B1;1 protein as represented by SEQ ID NO: 2, or a homologue, derivative or active fragment thereof.

35 36. Construct according to claim 34, wherein said nucleic acid of (a) is a CDK B1;2 nucleic acid as represented by SEQ ID NO: 3 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, which nucleic acid encodes a CDK B1;2 protein as represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof.

103-CDKB-PROV

37. Construct according to claim 34, wherein said nucleic acid of (a) is a CDK B2;2 nucleic acid as represented by SEQ ID NO: 5 or by a portion thereof, or by a nucleic acid sequence capable of hybridising therewith, which nucleic acid encodes a CDK B2;2 protein as represented by SEQ ID NO: 6, or a homologue, derivative or active fragment thereof.
38. Construct according to claim 34 or 35, wherein said control sequence comprises a promoter active in young, expanding tissue, preferably wherein said promoter is a beta expansin promoter.
39. Construct according to claim 34, 36 or 37, wherein said control sequence comprises a constitutive promoter, preferably a GOS 2 promoter.
40. Method for the production of a transgenic plant having modified growth characteristics, which method comprises:
- (a) introducing into a plant or a plant cell a nucleic acid capable of modulating expression of a B-type CDK gene/nucleic acid and/or activity and/or levels of a B-type CDK protein; or
 - (b) a nucleic acid encoding a CDK mutant, which CDK mutant comprises at least one of the seven amino acid position changes shown in Table A;
 - (c) cultivating the plant cell under conditions promoting regeneration and mature plant growth.
41. Transgenic plant having modified growth characteristics, characterised in that said plant has modulated expression of a B-type CDK nucleic acid and/or modulated activity and/or levels in a plant of a B-type CDK protein.
42. Transgenic plant according to claim 41, wherein said plant is a monocotyledonous plant, preferably a cereal, such as rice, maize, wheat, millet, barley.
43. Use of a CDK B-type nucleic acid or use of a CDK B-type amino acid in modifying the growth characteristics of plants.
44. A composition comprising a protein represented by SEQ ID NO 2, SEQ ID NO: 4 or SEQ ID NO: 6 or a homologue, derivative, or active fragment of any of the aforementioned sequences, for the use as a growth regulator.

103-CDKB-PROV

Abstract**Plants having modified growth characteristics and method for making the same**

5

The present invention concerns a method for modifying the growth characteristics of plants by modulating expression in a plant of a B-type CDK nucleic acid and/or modulating activity and/or levels in a plant of a B-type CDK protein. The invention also relates to transgenic plants having modified growth characteristics, which plants have modulated expression of a B-type CDK nucleic acid and/or modulated activity and/or levels in a plant of a B-type CDK protein relative to expression, activity and/or levels in corresponding wild-type plants. The present invention also provides a novel screening method for the identification of mutant CDKs having enhanced CDK activity relative to corresponding non-mutated CDKs. The present invention also provides a novel screening method for the identification of non-active CDKs that are still able to bind to CKIs (cyclin dependent kinase inhibitors). The invention also provides mutant CDKs obtainable by the screening methods according to the invention.

20

103-CDKB-PROV

1/10

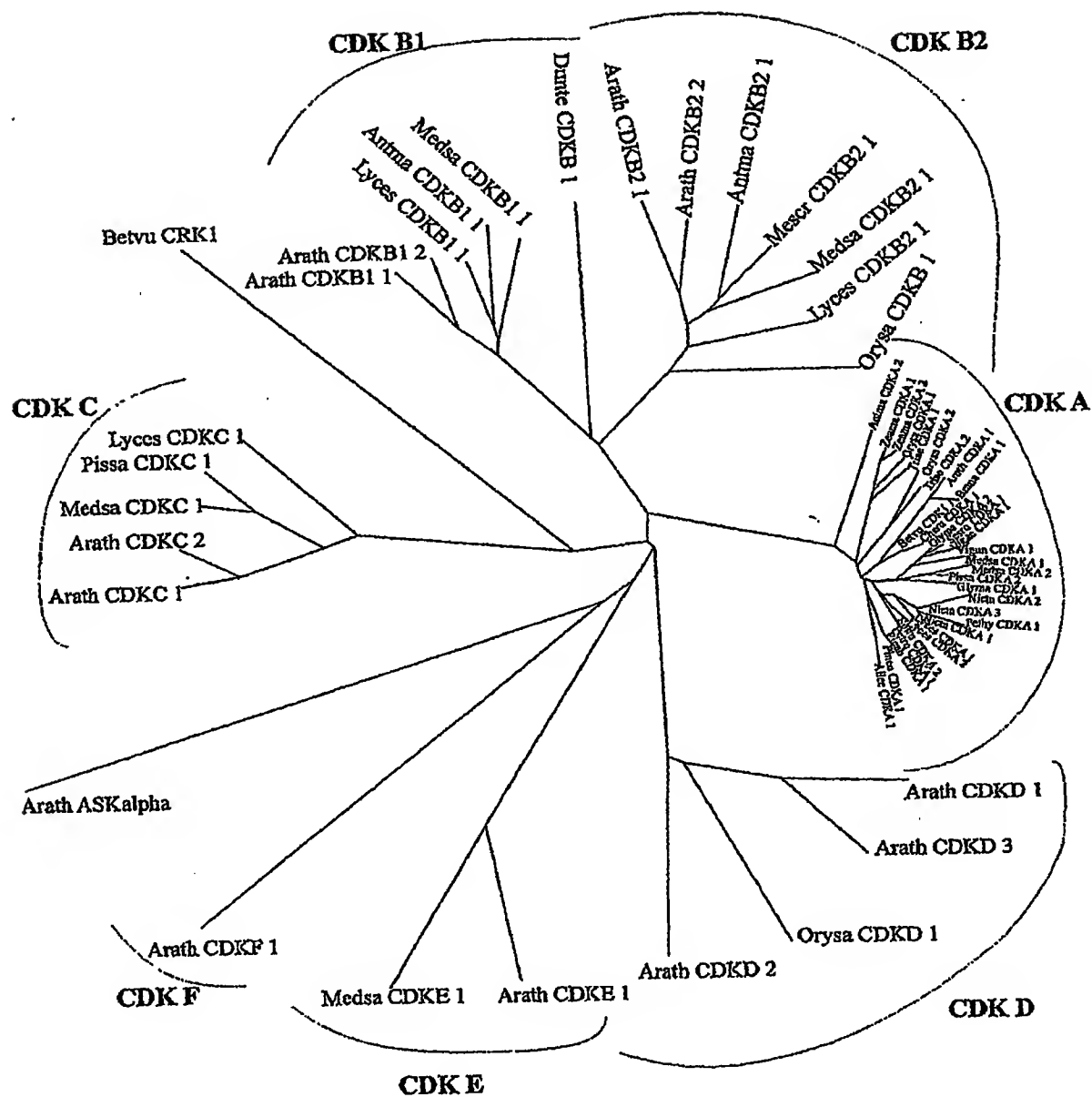


FIGURE 1

103-CDKB-PROV

2/10

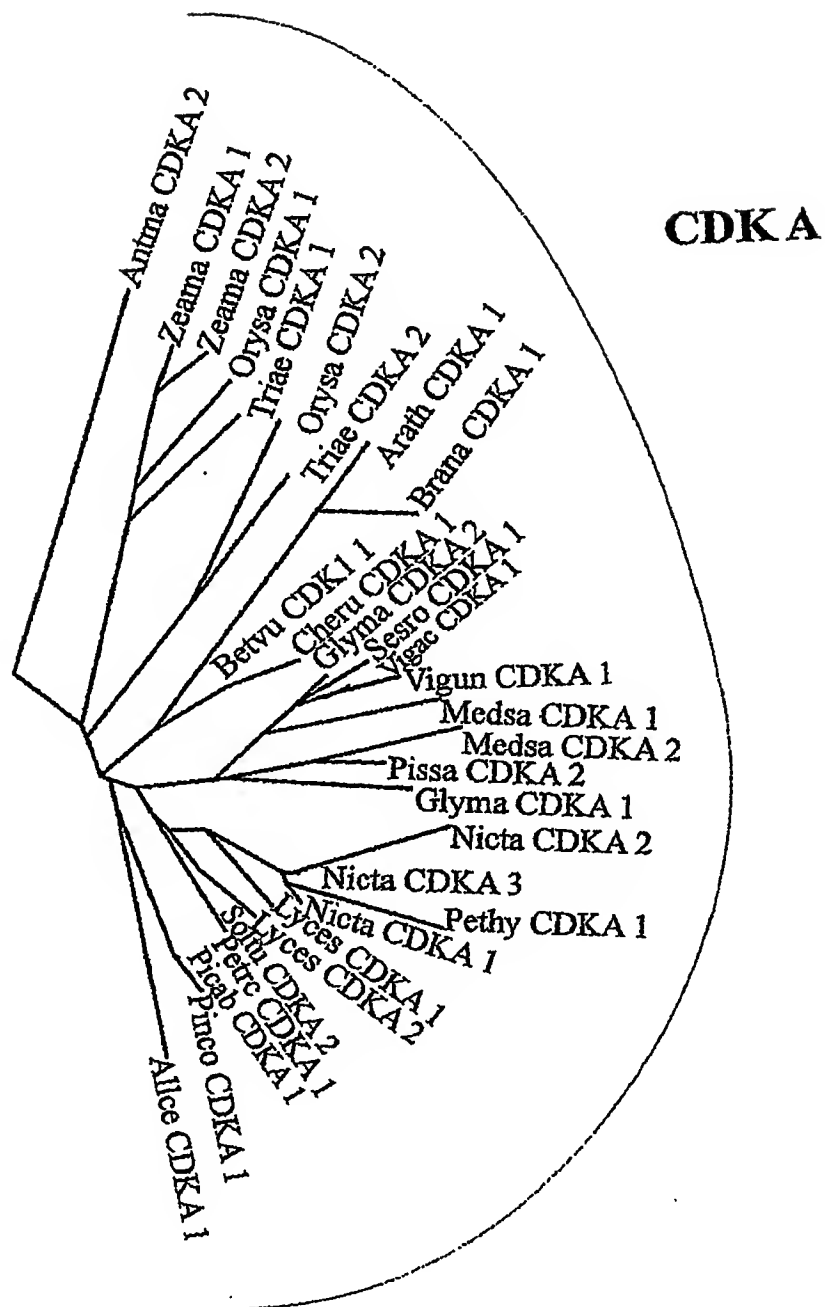


FIGURE 2

J3-CDKB-PROV

3/10

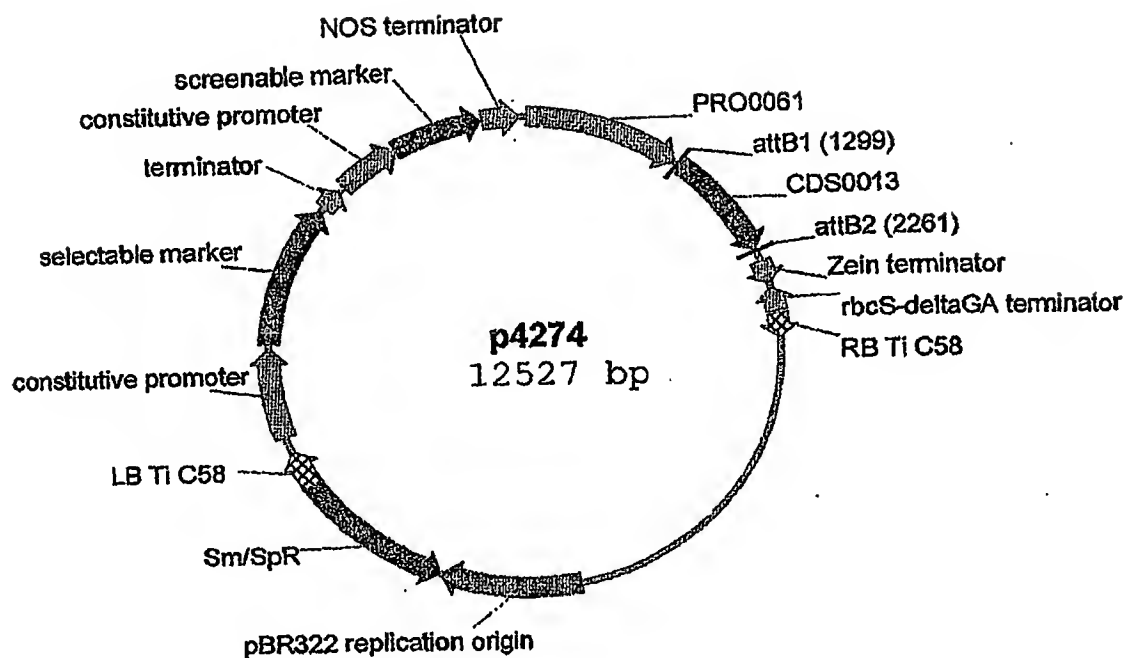


FIGURE 3

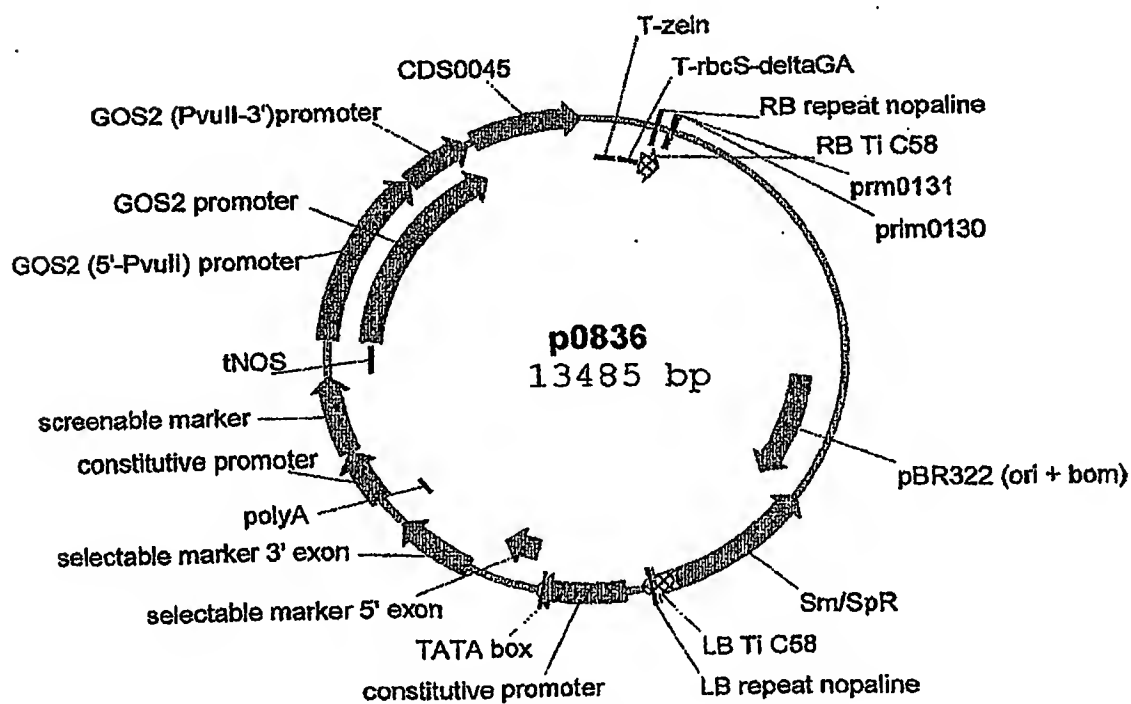


FIGURE 4

103-CDKB-PROV

4/10

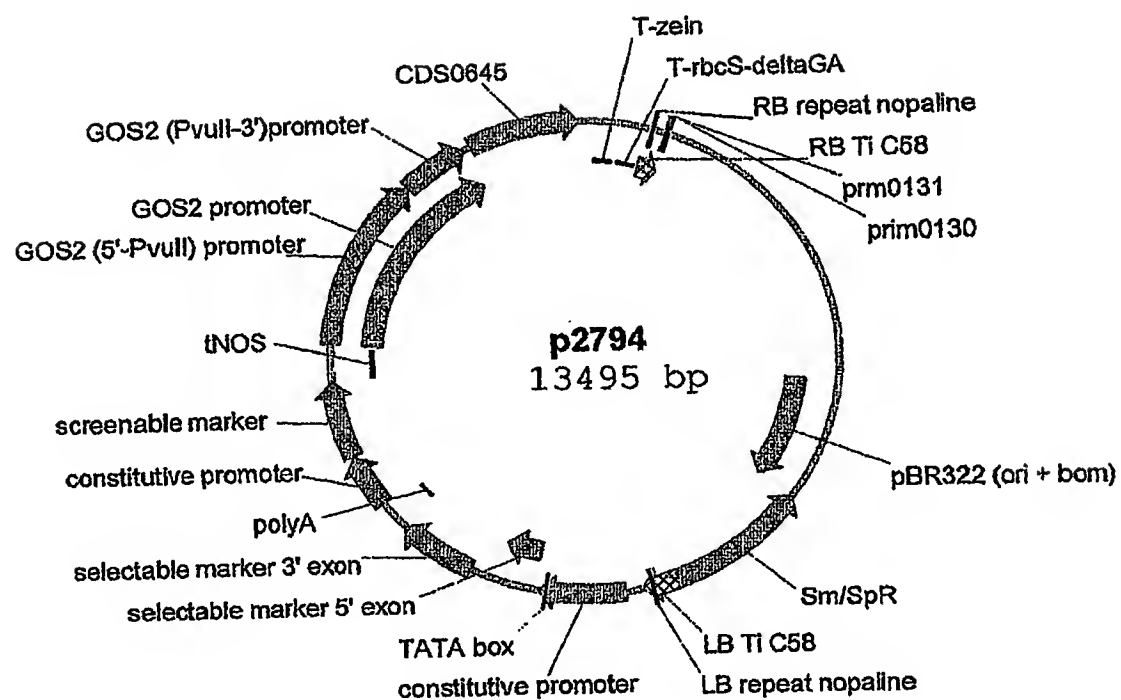


FIGURE 5

103-CDKB-PROV

5/10

SEQ ID NO 1: DNA sequence of CDK B 1;1

atggagaagtaacgagaagctagagaaggtcggagaaggaacatacgggaaagtctacaaagc
gatggagaaggaactggtaagcttggtgctctgaagaaaactcgtctcgagatggacgaag
aaggattccaccaactgctcttcgtgagatctcgtcttccagatgttatcaacatogatc
tatgttggttcgattactctgctcgaacatgttcatcaaccatcaaccaaattctcaatctac
caaatccaatctctatctcgttttcgagtatctcgatactgatcttaagaaattcatcgatt
cgtataggaaaggacctaatacctaagcctcttgagccttttttgattcagaagttgatgtt
cagctttgtaaaggtgttgcgcatgtgtcatagtcattggtgtgcttcaccgtgatcttaaac
gcagaatcttctctggtgaaagataaagagcttcttaagattgctgatttgggtcttggtc
gtgcttttactgttctcttaagtcttatacgcagatgattgttactctttggtatagagct
cctgaagttcttcttggtatctactcattattcaactggtgttgacatgtggtctgttggtg
tatctttgctgagatgggtcggaggcaagctctttccctggtgattctgagtttcagcaat
tgcttcataatcttcaggttgctaggaacaccaactgagcagcaatggccgggtgttccaca
ctgcgtgactggcatgtttaccctaagtgggagccgcaagacttaactcttgctgttccttc
tctttcacctcaaggagttgatcttctcacgaaaatgctcaagtacaatccagccgaaagaa
tttcagcaaaaacagcacttgatcaccatattttgacagccttgacaagtctcagttctga

SEQ ID NO 2: Protein sequence of CDK B 1;1

MEKYEKLEKVGEGTYGKVYKAMEKGTGKLVALKKTRLEMDEEGIPPTALREISLLQMLSTSI
YVVRLLCVEHVHQPSTKSQSTKSNLYLVFEYLDLTKKFIDSYRKGNPKPLEPFLIQKLMF
QLCKGVAHCHSHGVLHRDLKPQNLVLDKELLKIADLGLGRAFTVPLKSYTHEIVTLWYRA
PEVLLGSTHYSTGVDWMSVGCIFAEMVRRQALFPDSEFQQLLHIFRLGTPTEQQWPGVST
LRDWHVYPKWEPODLTLAVPSLSPOGVDLLTKMLKYNPAERISAKTALDHPYFDSLDKSQF

SEQ ID NO 3: DNA sequence of CDK B 1;2

atggagaaatacagagaagctcgaagaggtcgggtgaaggaacctatggaaaagtctacaaagc
aatggagaaaaccaccggaaaactcgtcgtctgaagaaaactaggctcgaaatggacgaag
aaggatataccaccaacggctctccgtgagatctctctctccaaatgctttctcaatcaatc
tacatcgttcgtctcctctgctcgaacatgttattcaatcgaagattcgactgtttctca
ctctcccaaatccaatctctatctcgtttttgagtatctcgacactgatctcaagaaattta
tagattctcatagaaagggctcgaatcctagaccgcttgaggcttctcttggtgcagaggtt
atgtttcagcttttttaaaggtgtggtcattgtcatagccatgggtgtgcttcaccgtgatct
taaaccgcagaatcttctattggataaaggataaagggtattcttaagattgctgatttgggtc
ttagtcgtgcttttactgtgcctcttaaggcttatacacatgagattgttactctttggtat
agagctcctgaagttttgcttggttctactcattactctactgctgttgatatttggtctgt
tggtatgcattctttgccgagatgattaggaggcaagctctttccctggtgattctgagtttc
agcaactacttcatattttcagattgttaggaacaccaactgagcagcaatggccgggtgta
atggcattgcgtgactggcatgtctatccaaagtgggagccgcaagacttatcacgtgctgt
tccatctctatctcctgaaggaattgatcttctcacgaaaatgttgagtaacaatccagcag
aaagaatttcagcaaaagcagctctcgatcatccctactttgacagccttgacaaatctcag
ttctga

FIGURE 6

103-CDKB-PROV

6/10

SEQ ID NO 4: Protein sequence of CDK B 1;2

MEKYEKLEKVGEGTYGKVKAMEKTTGKLVALKKTRLEMDEEGIPPTALREISLLQMLSQSI
YIVRLLCVEHVIOQKSTVSHSPKSNLYLVFEYLDLKKFIDSHRKGSNPRPLEASLVQRF
MFQLFKGVAHCHSHGVLHRDLKPQNLLDKDKGILKIADLGLSRAFTVPLKAYTHEIVTLWY
RAPEVLLGSTHYSTAVDIWSVGCIFAEMIRQALFPGDSEFQQLLHIFRLLGTPTEQQWPGV
MALRDWHVYPKWE PQDLSRAVPSLSPEGIDLLTQMLKYNPAERISAKAALDHPYFDSLDKSQ
F

SEQ ID NO 5: DNA sequence of CDK B 2;2

atggacaacaatggagttaaacccgctgtttccgccatggaagcctttgaaaagcttgagaa
agtaggtgaagggacttatgggaaagtttacagagcaagagagaaagctactgggatgatcg
ttgctttgaagaagacgcgtctccatgaggatgaagaagggtgtccctccactactcttcgc
gagatctctatcttgctgtatgctcgctcgtgatccctcacatcgtaggtgatggatgttaa
gcaaggaataaacaagaaggaaaaactgtactttaccttggtttogagtatgttgatactg
atctcaagaaattcatcagaagctttcgtcaagctggacagaacattccacaaaatactgtc
aagtgttgatgtaccagttatgcaaaggcatggcttttgccatgggtcatggagtgttgca
cagggatcttaagcctcacaatctcttgatggaccggaagacaatgacgctcaaaatagcag
atcttggttagcagagccttactctcccaatgaaaagatacacatgagattctaact
ctatggtatagagctccggaagtctctcttgagcaaccattactctactggagtggatat
gtggtctgttggtgtatttttgctgaactagtgaaccaagcaagcaatctttgcgggagact
ctgagctccaacagctcctccgtatattcaggttggtgggaacaccaaacgaagaagtttg
cctggagtaagcaaaactcaaggactggcatgaatacccgcaatggaaacggttgagctcttc
cacagctgtgccaacctcgacgaggctggacttgatctcttatctaaaatgctggagtacg
agccagcaaaacgaatctcagcaaaagaaagctatggagcatccttacttcgatgatttgct
gacaagtcctctctctga

SEQ ID NO 6: Protein sequence of CDK B 2;2

MDNNGVKPAVSAMEAFEKLEKVGEGTYGKVYRAREKATGMIVALKKTRLHEDEEGVPPTTLR
EISILRMLARDPHIVRLMDVKQGINKEGKTVLYLVFEYVDTDLKKFIRSFQAGQNIPTQTV
KCLMYQLCKGMAFCHGHGVLHRDLKPHNLLMDRKTMTLKIADLGLARAFTLPMKKYTHEILT
LWYRAPEVLLGATHYSTGVDMWSVGCIFAELVTKQAFAGDSELQQLLRIFRLLGTPNEEVW
PGVSKLKDWHYEPQWKPLSLSTAVPNLDEAGLDLLSKMLEYEPKRISAKKAMEHPYFDDL
DKSSL

FIGURE 6 (continued)

103-CDKB-PROV

7/10

SEQ ID NO 7: DNA sequence of CDK A;1 (*Oryza sativa*)

acctctcctccgattaatccccctccccctcctcttctcctccacttctgcgctgctcttctcctc
ccctcgccgaccctacctactcgcgccgcccgcgtcgattggggcggaacacggagggggggg
ttaaccctgatggagcagtagcagaaggaggagaagattggggagggcacgtacggggtggt
gtacagggcgcgggacaaggtcaccaacgagacgatcgcgctcaagaagatccggcttgagc
aggaggatgagggcgctccccctccaccgcaatccgcgagatctcgctcctcaaggagatgcat
cacggcaacatcgctcaggttacacgatgttatccacagtgagaagcgcatatatcttgtctt
tgagtatctggatctggaacctaaagaagttcatgtgactcttgtccagagtttgcgaaaaacc
ccactttaattaagtcatatctctatcagatactccgcgcggttgcttactgtcattctcat
agagttcttcatcgagatttgaaacctcagaatttattgatagatcggcgtagtaactgcact
gaagcttgcaacttttggtttagccaggcgatttgaatttctgtccgcacgtttactcacg
aggttgtaaccttggtgtatagagctccagagatccttcttggtcaaggcagttattctaca
ccagttgatattgtggtcagttggtgtatctttgcagaaatggtgaaccagaaaccactggt
ccctggtgattctgagattgatgaattatttaagatattcagggtactaggaactccaaatg
aacaaagttggccaggagttagctcattacctgactacaagctgctgtttccccaagtggcaa
gcacaggtatcttgcaactattgtccctactcttgaccctgctggtttggaccttctctctaa
aatcggttcggtacgagccaaacaaaaggatcacagctagacaggctcttgagcatgaatact
tcaaggaccttgagatggtacaatgacctgctatggctttacattggattggcatatgtat
gggctgggctcctcatttcattccttctgtgaacgctgtgcccttcggttgggcatttttg

SEQ ID NO 8: Protein sequence of CDK A;1 (*Oryza sativa*)

MEQYEKEEKIGEGTYGVVYRARDKVTNETIALKKIRLEQEDEGVPSTAIREISLLKEMHAGN
IVRLHDVHSEKRIYLVFEYLDLDLKKFMDSCPEFAKNPTLIKSYLYQILRGVAYCHSHRVL
HRDLKPQNLLIDRRTNALKLADFLARAFGIPVRTFTEHVVTLLWYRAPEILLGSRQYSTPVD
MWSVGCIFAEMVNQKPLFPDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAPFKWQAQD
LATIVPTLDPAGLDLLSKMLRYEPNKRITARQALEHEYFKDLEMVQ

SEQ ID NO 9: Protein sequence of Mutant 1 of CDK A;1

MEQHEKEEKIGEGTYGVVYRARDKVTNETIALKKIRLEQEDEGV PSTAIREISLLKEMHHGN
IVRLHDVHSEKRIYLD FEYLDLDLKKFMDSCPEFAKNPTLIKSYLYQILRGVAYCHSHRVL
HRDLKPQNLLIDRRYTNALKLADFG LARTFGIPVRTF THEVVTLWYRAPEILLGSRQYSTPVD
MWSVGCIFAEMVNQKPLFP GDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAPFKWQAQD
LATIVPTLDPAGLDLLSKMLRYEPNKRI TARQALEHEYFKDLEMVQ

SEQ ID NO 10: Protein sequence of Mutant 2 of CDK A;1

MEQYEKEEKIGEGTYGVVYRARDKVTNETTALKKIRLEQEDEGVPSTAIRESLLKEMHHGN
IVRLHDVIHSEKRIYLVFEYLDLDLKKFMDSCPEFAKNPTLIKSILYQILRGVAYCHSHRVL
HRDLKPQNLLIDRRTNALKLADFGRLARAFGIPVRTFTHEVVTLWYRAPEILLGSRQYSTPVD
MWSVGCIFAEMVNQKPLFPDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAPFKWQAQD
LATIVPTLDPAGLDLLSKMLRYEPNKRITARQALEHEYFKDLEMVQ

FIGURE 6 (continued)

103-CDKB-PROV

8/10

SEQ ID NO 11: Protein sequence of Mutant 3 of CDK A;1

MEQYVKEEKIGEGTYGVVYRARDKVTNETIALKKIRLEQEDEGVPSTAIRESLLKEMHHGN
IVRLHDVIHSEKRIYLVFEYLDLDDLKKFMDSCPEFAKNPTLIKSYLYQILRGVAYCHSHSVL
HRDLKPQNLLIDRRTNALADFLARAFGIPVRTFTHEVVTWYRAPEILLGSROYSTPVD
MWSVGCIFAEMVNQKPLFPDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAFPKWQAQD
LATIVPTLDPAGLDLLSKMLRYEPNKRITARQALEHEYFKDLEMVQ

SEQ ID NO 12: Protein sequence of Mutant 4 of CDK A;1

MEQYEKEEKIGEGTYGVVYRARDKVTNETIALKKIRLEQEDEGVPSTAIRESLLKEMHHGN
IVRLHDVIHSEKRIYLVFEYLDLDDLKKFMDSCPEFAKNPTLIKSYLYQILRGVAYCHSHSVL
HRDLKPQNLLIDRRTNALKLADFLARAFRIPVRTFTHEVVTWYRAPEILLGSROYSTPVD
MWSVGCIFAEMVNQKPLFPDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAFPKWQAQD
LATIVPTLDPAGLDLLSKMLRYEPNKRITARQALEHEYFKDLEMVQ

SEQ ID NO 13: Protein sequence of Mutant 5 of CDK A;1

MEPYEKEEKIGEGTYGVVYRARDKVTNETIALKKIRLAQEDEGVPSTAIRESLLKEMHHGN
IVRLHDVIHSEKRIYLVFEYLDLDDLKKFMDSCPEFAKNPTLIKSYLYQILRGVAYCHSHSVL
HRDLKPQNLLIDLRTNALKLADFLARAFGIPVRTFTHEVVTWYRAPEILLGSROYATPVD
MWSVGCTFAEMVNQKPLFPDSEIDELFKIFRVLGTPNEQSWPGVSSLPDYKSAFPKWQAQD
LATIVPTLDPAGLDLLSKVLRYEPNKRITARQALEHEYFKDLEMVQ

SEQ ID NO 14: DNA sequence of beta-expansin promoter EXPB8

aaaaccaccgaggacgtgatctgcaccggttttgatagttgagggacccgttggtgtctggt
tttccgatcgaggagcgaataatcggttcggtgtaaagttaaggacactcagatgaacttat
tccggagcatgattgggaaggaggacataaggccatgtcgcgtgtgttggaagggtccag
atctccagatcactcagcaggatcgccgcgttcgcgtagcaccgcggtttgattcggctt
cccgcaaggcgccggtggccgtgcccgcgtagcttccgcccgaagcagcagcgcgcgc
gcccgcgaccggtctctgcgtttgcaccgccttgacgcgatacatcgggatagatagctac
tactctctccggtttcacaatgtaaatcattctactatttccacattcatattgatgtaat
gaatatagacatatatatctatttagattcattaacatcaatatgaatgtaggaaatgctag
aatgacttacattgtgaattgtgaaatggacgaagtacctacgatggatggatgcaggatca
tgaaagaattaatgcaagatcgtatctgccgcatgcaaaatcttactaattgcgctgcatat
atgcatgacagcctgcatgcggcggtgtaagcgtgttcattccattaggaagtaacctgtca
ttacttataccagtactacatactatatagattgatttcattgagcaaatctacaaaactgg
aaagcaataagaaatacgggactggaaaagactcaacattaatcaccaaatatttcgccttc
tccagcagaatatatatctctccatcttgatcactgtacacactgacagtgtacgcataaac
gcagcagccagcttaactgtcgtctcaccgctgcacactggccttcattctcaggctagctt
tctcagccaccatcgatcatgtcaactcggcgcgcgacaggcacaaattacgtacaaaac
gcatgaccaaatacaaaaccacggagaagaatcgctcccgcgcgcgccggcgacgcgcacgt
acgaacgcacgcacgcacgcccacccacgacacgatcgcgcgcgacgcggcgacacccgg
ccgtccaccgcgcgcctcacctcgccgactataaatacgtaggcatctgcttgatcttgtca
tccatctcaccaccaaataaaagggaataaaacacaccaaagccaaataaaagcg
aca

FIGURE 6 (continued)

103-CDKB-PROV

9/10

SEQ ID NO 14: DNA sequence of GOS2 promoter

aatccgaaaagtttctgcaccgttttcacccttaactaacaatatagggaaacgtgtgctaa
atataaaatgagaccttatatatgtagcgctgataactagaactatgcaagaaaaactcatc
cacctacttttagtggcaatcgggctaaataaaaaagagtcgctacactagtttcgttttcct
tagtaattaagtgggaaaatgaaatcattattgcttagaatatacgttcacatctctgtcat
gaagttaaattattcgaggtagccataattgtcatcaaactcttcttgaataaaaaaatctt
tctagctgaactcaatgggtaagagagagattttttttaaaaaatagaatgaagatattc
tgaacgtattggcaaagatttaaacatataattatataattttatagtttgtgcattcgta
tatcgacatcattaaggacatgtcttactccatcccaatttttatttagtaattaaagaca
attgacttatttttattatttattcttttttcgattagatgcaaggtaacttacgcacacactt
tgtgctcatgtgcatgtgtgagtgacacctcctcaatacacgttcaactagcaacacatctct
aatatcactcgcctattttaatacatttaggttagcaatatctgaattcaagcactccaccatc
accagaccacttttaataatatctaaaatacaaaaaataattttacagaatagcatgaaaag
tatgaaacgaactatttaggtttttcacatacaaaaaaagaattttgctcgtgcgcga
gogccaatctcccatattgggcacacaggcaacaacagagtggtgcccacagaacaacca
caaaaaacgatgatctaacggaggacagcaagtcgcaacaaccttttaacagcaggctttg
cgccaggagagaggaggagagcaagaaaaaccaagcatcctcctcctccatctataaat
tcctcccccttttccctctctatataggaggcatccaagccaagaagaggagagacca
aggacacgcgactagcagaagccgagcgaccgcttcttcgatccatacttccggctcgagt
tcttggtcgatctcttccctcctcctcctcacagggtatgtgcccttcggttggtc
ttggatttattgttctaggttggtgtagtacggcggttgatgttaggaaaggggatctgtatc
tgtgatgattcctgttcttggatttgggatagaggggttcttgatgttgcatgttatcggtt
cggtttgatttagtagtatggttttcaatcgctctggagagctctatggaaatgaaatggttta
gggtacggaatcttgcgattttgtgataccttttgggtgaggtaaaaatcagagcaccggtga
ttttgcttggtgtaataaaagtacggttggttggtcctcgattctggttagtgatgcttctcg
atttgacgaagctatcctttgtttattccctattgaacaaaaataatccaactttgaagacg
gtcccggtgatgagattgaatgattgattcttaagcctgtccaaaatttcgcagctggcttg
tttagatacagtagtccccatcacgaaattcatggaaacagttataatcctcaggaacaggg
gattccctgttcttccgatttgccttagtcccagaatttttttcccaaatatcttaaaaag
tcaactttctggttcagttcaatgaattgattgctacaaataatgcttttatagcggtatcct
agctgtagttcagttaataggtaataccctatagtttagtcaggagaagaacttatccgat
ttctgatctccatttttaattatataatgaatgaactgtagcataagcagattcatttggatt
attttttttttagctctcacccttctcattattctgagctgaaagtctggcatgaactgtcct
caattttgttttcaaattcacatcgattatctatgcattatcctcttgtatctacctgtaga
agtttctttttggttattccttgactgcttgattacagaaagaaatttatgaagctgtaac
gggatagttatactgcttgttcttatgattcatttctttgtgcagttcttgggtgtagcttg
ccactttcaccagcaaagttc

SEQ ID NO 15: DNA sequence of sense primer prm0350

ggggacaagtttgtacaaaaaagcaggcttcacaatggagaagtagcagaagctaga

FIGURE 6 (continued)

103-CDKB-PROV

10/10

SEQ ID NO 16: DNA sequence of antisense primer prm0351

ggggaccactttgtacaagaaagctgggttcagaactgagacttgtcaagg

SEQ ID NO 17: DNA sequence of sense primer prm439

ggggacaagtttgtacaaaaaagcaggcttcacaatggagaaatacgagaagctc

SEQ ID NO 18: DNA sequence of antisense primer prm440

ggggaccactttgtacaagaaagctgggtggtcagaactgagatttgtc

SEQ ID NO 19: DNA sequence of sense primer prm2213

ggggacaagtttgtacaaaaaagcaggcttcacaatggacaacaatggagttaa

SEQ ID NO 20: DNA sequence of antisense primer prm2214

ggggaccactttgtacaagaaagctgggttcagagagaggacttgtcag

FIGURE 6 (continued)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.